Lipid composition of Saccharomyces cerevisiae DCL 740 in relation to morphogenesis of ascospores.

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LIPID COMPOSITION OF
Saccharomyces cerevisiae DCL 740
IN RELATION TO MORPHOGENESIS OF ASCOSPORES

submitted by

ROBERT FRANK ILLINGWORTH

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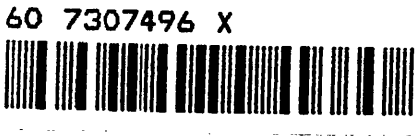
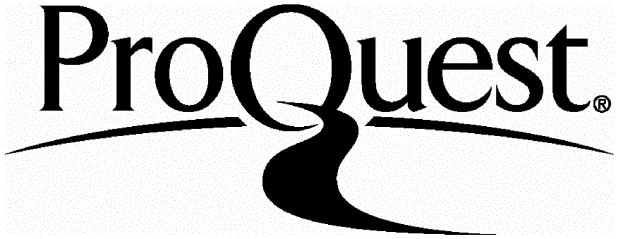
R. F. Illingworth.





SUMMARY

Approximately 80% of vegetative cells of Saccharomyces cerevisiae form asci, the majority of which are four-spored, when incubated in a suitable sporulation medium. Ascospore delimitation is initiated by a pair of unit membranes, the spore-delimiting membranes, between which the spore wall is eventually laid down. Numerous lipid vesicles are observed to be in close association with the spore-delimiting membranes during delimitation and spore wall formation. Their role during these processes is discussed. The dry weight of cells increases by about 75% during sporulation while the lipid content increases by a factor of four. The increase in lipid content is attributable to increased synthesis of sterol esters, triacylglycerols and phospholipids. Uniformly labelled [^{14}C] acetate is incorporated mainly into sterol esters, triacylglycerols and phospholipid. Pulse-labelling by adding [U-^{14}C] acetate to sporulating cultures and harvesting after a further six hours incubation reveals two main periods of lipid synthesis, namely between T_0 and T_{18}, and between T_{24} and T_{30}. The composition of sterols and phospholipids does not change appreciably, but there is a marked increase in the proportion of unsaturated fatty acids. The significance of these changes in lipid content and composition are discussed in relation to the appearance of the spore-delimiting membranes, endoplasmic reticulum membranes and lipid vesicles. Electrophoretic measurements on individual ascospores indicate the presence of a surface protein layer. Inhibition of ascosporogenesis by ammonium ions was investigated. Asci from cultures in the period T_{20} to T_{30} were found to be particularily susceptible to inhibition by ammonium ions.

CONTENTS

Briley, M S, R F Illingworth A H Rose and D J Fisher. 1970. Evidence for a surface protein layer on the Saccharomyces cerevisiae ascospore. J.Bacteriol. 104:588-589.

Illingworth, R F, A H Rose and A Beckett. 1973. Changes in the lipid composition and fine structure of Saccharomyces cerevisiae during ascospore formation. J. Bacteriol. 113:373-386.

Beckett, A, R F Illingworth and A H Rose. 1973. Ascospore wall development in Saccharomyces cerevisiae. J. Bacteriol. 113:1054-1057.

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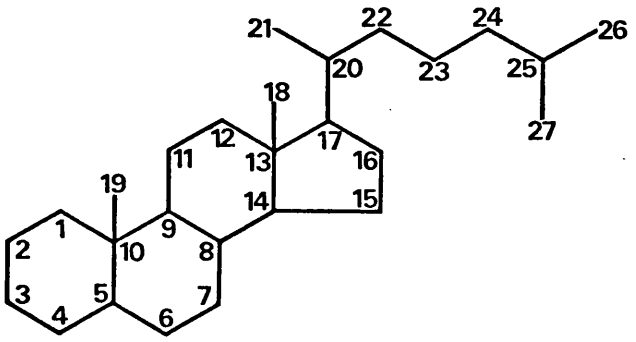
ABBREVIATIONS

Fatty acids. These are referred to as $C_{x:y}$ where x indicates the number of carbon atoms in the straight chain fatty acids and y indicates the number of double bonds in the chain.

Sterols. Δ^z indicates the position of a double bond in the sterol structure; the numbering is as shown. The double bond is between carbon atom z and z+1.



All temperatures recorded in this thesis are in degrees Celsius.

SDS is the abbreviation for sodium dodecyl sulphate.

The abbreviations for all other chemical compounds and the abbreviations for the names of the Journals referred to in the Bibliography are those recommended by the Biochemical Journal (1973; 131:1-20).

ACKNOWLEDGEMENTS

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I would like to express sincere thanks to my parents whose constant encouragement has sustained me during the pursuit of my educational aspirations.

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INTRODUCTION

The term 'yeast' embraces a very heterogenous group of microorganisms. It is a very popular and widely used term, although the concept of yeast is due more to historical development than phylogenetic considerations.

Vegetative reproduction in yeasts is usually by budding. A small swelling appears on the cell wall of the parent cell, generally near to the poles, and develops into a daughter cell. This eventually separates from the mother cell. Yeasts in two genera, Endomyces and Schizosaccharomyces reproduce exclusively by fission. In this case a cross-wall forms without constriction of the cell wall and then divides into two individual cross-walls.

Morphology. The predominant morphological form of yeast is a single cell or usually a single cell with an attached bud. However, buds may continue to form without breaking away from the parent cell; this leads to the formation of clusters and chains of cells. Chain formation can lead to the formation of pseudomycelia which arise when, instead of breaking away at maturity, the bud remains attached to the mother cell, elongates and continues to bud in turn. In this manner strands are formed which resemble a true mycelium, (cells separated by a cross-wall) differing only in the manner in which new cells arise (budding). The process of pseudomycelium formation occurs in some yeasts, eg Candida spp. under certain growth conditions. Some fission yeast are able to produce true mycelial hyphae which contain cross walls at regular intervals. These hyphae may break up into separate cells termed arthrospores, as in Trichosporon; or the hyphae may produce

budding cells as in Endomycopsis. A general review of yeasts and their morphology is given by Phaff, Miller and Mrak (1966).

Taxonomy. Fungi are divided into four classes, and yeasts are grouped within three of the four classes. The first and largest group of yeasts is characterised by the formation of ascospores within a modified cell or ascus. These yeasts, therefore, belong to the Ascomycetaceae. The second group is classified in the family Sporobolomycetaceae. They produce ballistospores and belong to the Basidiomycetaceae. Yeasts of the third group have not yet been observed to pass through a sexual phase or to produce ascospores or ballistospores. This group is part of the Fungi Imperfecti. The Phycomycetes do not have any recognised yeast representatives. For a review of yeast taxonomy and phylogeny, see Lodder (1970).

Various criteria connected with ascospores have been used in taxonomic and systematic studies of ascogenous yeasts. These criteria have included the shape of the ascus, the shape of the ascospores, the surface features and colour of the ascospores and the number of ascospores per ascus. Spherical or globose spores with a smooth surface are found in Saccharomyces species and various other genera. Spores with a kidney-bean shape are characteristic of Fabospora and Shizosaccharomyces octosporus. Spores of yeasts in several other genera possess an encircling brim; the position of the brim and its prominence on the spore varies. Spores of Pichia are helmet-shaped; spores of Hansenula, which possess a more prominent brim, are hat-shaped. Spores of Hansenula saturnus possess an equatorial brim and hence are saturn-shaped. Needle-like

spores are produced by strains of Coccidiascus, Monosporella and Nematospora.

The asci of many yeasts contain a maximum of four spores. A few yeasts, however, such as Shizosacch. octosporus and Nematospora spp., resemble higher Ascomycetes and regularly produce eight-spored asci. Some strains of Sacch. cerevisiae tend to give rise to eight-spored asci (Winge and Roberts, 1950). Kluyveromyces polysporus forms asci which contain an estimated 100 spores (van der Walt, 1956).

Spores of Nadsonia spp. and Lipomyces spp. appear brownish in colour even under the light microscope. Sporulating cultures of Fabospora and some species of Pichia and Hansenula appear pink to red-brown.

Reduction division. The true significance of ascospore formation in yeast was discovered by Kruis and Satava (1918) and independently, much later, by Winge (1935) and Winge and Laustsen (1937). These workers observed several phenotypic characters, including colony size, cell morphology, fermentative ability, to segregate independently during spore formation. These investigations showed that most strains of Sacch. cerevisiae (Winge and Laustsen, 1937) and Saccharomycodes ludwigii (Winge and Laustsen, 1939b) are heterozygous. If spores from two different strains of Sacch. cerevisiae or two different species of Saccharomyces are placed together, conjugation followed by nuclear fusion may occur (Winge and Laustsen, 1939a). Hybrids formed are diploid and heterozygous. These experiments demonstrated that fermentative ability is a dominant character.

These observations established that vegetative cells of Sacch. cerevisiae are diploid and that sporulation involves a meiotic division which produces haploid ascospores. This discovery of alternation of diplophase and haplophase opened up the possibility of extensive genetic studies with yeasts. Tetrad analysis of asci provides information about the genetic control of various characteristics, and enables maps of linkage groups to be constructed. Cultures arising from single ascospores can be used for studies on segregation and mutation (Mortimer and Hawthorne, 1969).

Cultures from single ascospores are also employed in hybridisation programmes designed to improve the strains of Sacch. cerevisiae used in the brewing, baking and distilling industries. Spores, or haploid cultures, termed 'maters' from strains noted for particular characters, (such as speed of fermentation, substrates utilised, flocculation ability) are crossed and the hybrids selected for their ability to express this type of character (Fowell, 1969).

Physiology of sporulation

It is generally believed that sporulation in yeasts was first reported by Schwann in 1839. He observed yeast cells which had formed "smaller interior cells". These smaller cells were released when the parent cell burst.

A wealth of information is available about sporulation in Sacch. cerevisiae, and all further discussion of sporulation in this thesis will refer exclusively to this organism, unless specifically stated otherwise. The literature is especially well documented with data

about factors which predispose cells to sporulation, and about conditions which are conducive to ascus formation. General reviews of sporulation in Sacch. cerevisiae and other yeasts are given by Phaff and Mrak (1948, 1949), Miller and Hoffmann-Ostenhof (1964), Fowell (1969) and Tingle, Klar, Henry and Halvorson (1973). However much less is known about the nature of initiation of sporulation, or about the nature of intracellular mechanisms which operate during induction, meiotic division or delineation of individual ascospores. Sporulation is divisible into two phases; the first is termed the 'presporulation' phase, the second the 'sporulation' phase. The presporulation phase is a period of vegetative growth, usually in a rich medium, referred to as the 'presporulation medium', immediately prior to the inducement of sporulation. The sporulation phase takes place in a replacement medium, the 'sporulation medium', which is unable to support vegetative growth by mitotic division, but which is able to induce meiosis and support the formation of ascospores.

Presporulation phase. Certain conditions in the presporulation phase must be fulfilled to prepare the cells for sporulation. This preparation involves establishment and maintenance of high concentrations of RNA and protein to support extensive biosynthetic activity and development of the ability for aerobic metabolism (Croes, 1967b). Many types of presporulation media have been devised which ensure adequate nourishment to predispose cells in this way. Media which contain a mixture of fruit and vegetable juices (Lindegren and Lindegren, 1944), tomato juice (Sando, 1956) or malt-extract wort (Fowell and Moorse, 1960) have been recommended as suitable

presporulation media.

Chemical constitutents which have been shown to be necessary include an assimilable carbohydrate (glucose is commonly employed), a source of nitrogen, and a full complement of B-group vitamins (provided by yeast extract or yeast autolysate). Abundant sporulation can be obtained using acetate as the carbon source in the presporulation medium (Roth and Halvorson, 1969). They reported optimum sporulation from cells which had been maintained in continuous log phase by repeated sub-culturing for at least 30h before transferring to sporulation medium. The presporulation medium which these workers recommend contains a yeast nitrogen base, yeast extract and potassium acetate, and was held at pH 5.0 by phthalate buffer.

The age of the presporulation culture has been shown to be an important factor. However, it must be realised that the optimum age of presporulation cells is governed by other factors such as the composition of the medium and the method of aeration. Fowell and Moorse (1960) found that cells were maximally predisposed toward sporulation after 40h incubation in a slowly stirred culture in malt-wort extract medium. Croes (1967b), using a medium containing glucose, a yeast nitrogen base and yeast extract, found that cells were optimally inducible after 18h.

Recent work has shown that the age of individual cells within a culture may be more important than the age of the culture itself. Mother and daughter cells differ in their sporogenic capacity (Yanagita, Yagisawa, Oishi, Sando and Suto, 1970). These workers

were able to differentiate between mother and daughter cells by fluorescent staining of bud scars, which are found only on mother cells. Cells which exhibited bud scars were observed to sporulate; cells which did not exhibit bud scars were observed not to sporulate. Cells taken from different stages of the cell cycle show different sporulation capabilities. Haber and Halvorson (1972) separated cells into three categories, on the basis of their size, by ultracentrifugation in sucrose density gradients. Single cells from the early stage in the cell cycle (cells recently formed by scission) sporulated poorly, cells from the middle stage sporulated reasonably well, but cells from the last stage of the cycle (cells with an attached bud) sporulated best of all.

Haber and Halvorson (1972) suggest that the capacity of cells to sporulate is limited during parts of the cell cycle by the availability of certain intracellular compounds. This idea is supported by Croes' (1967b) observation that the cellular content of RNA and protein affect the ability of cells to sporulate.

The effect of incubation temperature during the presporulation phase has not been extensively investigated, but Fowell and Moorse (1960) found that this parameter has little effect by itself.

Sporulation phase. Sporulation is essentially a response to starvation, an observation first made by de Seynes in 1868. Conditions in the sporulation phase need to reflect this requirement. The view that starvation is essential to inducement has been challenged by the fact that the presence of certain nutrients, albeit

in very small concentrations, are either necessary for sporulation or are required for optimum sporulation. However deprivation of most nutrients is still recognised as being the most important pre-requisite.

Many exotic media have been devised which fulfil the requirement for lack of nutrients. These have included, moistened blocks of gypsum (Engel, 1872) or cement (Hartelius and Ditlevsen, 1953), carrot or potato wedges, carrot-calcium sulphate agar (McKelvey, 1926) and agar containing a mixture of vegetable juices (Wickerham, Flickinger and Burton, 1946).

The most important ingredient of sporulation media has been found to be the carbon source. The presence of small concentrations of utilisable sugar promotes sporulation. The optimum concentration for glucose lies in the range 0.01% to 0.1% (w/v); concentrations higher than 1% (w/v) inhibit sporulation (Saito, 1916; Stantial, 1935). Salts of acetic acid are particularly effective for inducing a high degree of sporulation. The efficacy of acetate in promoting sporulation was reported for Saccharomyces carlsbergensis by Saito (1916) and for Sacch. cerevisiae by Stantial (1935). Sporulation media based on acetate were described by Adams (1949) and Fowell (1952) and this type of medium has now supplanted all others. Various types of acetate-containing media have been described by contemporary workers. These include 1% (w/v) potassium acetate (Chen and Miller, 1968; Roth and Halvorson, 1969; Moens, 1971; Guth, Hashimoto and Conti, 1972), 1% (w/v) potassium acetate plus 0.1% (w/v) yeast extract (Croes, 1967a; Esposito, Esposito, Arnaud and Halvorson, 1969), 0.5% (w/v) sodium acetate plus 1% (w/v) potassium

chloride (Fowell, 1967, 1969) and 0.98% (w/v) potassium acetate, plus 0.25% (w/v) yeast extract, plus 0.1% (w/v) glucose (Lynn and Magee, 1970).

Sporulation media which contain a nitrogen source, usually amino acids, but no carbon source are also able to promote sporulation (Fowell, 1955, 1967). However nitrogenous compounds depress or completely inhibit sporulation when present in media which contain a carbon substrate (Miller, 1963).

An important parameter which promotes optimum sporulation is the cell population density. This is the number of cells per unit volume of medium. The optimum cell population density is very dependent on the nature and concentration of the sporulation substrate, the degree of aeration, the presence or absence of agar in the medium and the pH value of the medium (Fowell, 1969). In liquid sporulation media which contain acetate and are vigorously stirred, the optimum cell population density lies between 1×10^6 and 1×10^7 cells per ml (Fowell, 1969).

Commitment to sporulation. Commitment is the point (in time) of no return for sporulating cells; once cells are committed to sporulation they are subsequently unable to revert to vegetative growth. In Gram-positive bacteria there is no single point of commitment for the whole sporulation process. Once bacterial cells have been initiated in a sporulation medium they become successively committed to one sporulation event after another (Mandlestam, 1971). It is probable that the same phenomenon occurs in Sacch. cerevisiae.

During sporulation of Sacch. cerevisiae various inhibitory compounds differ in the time at which commitment to sporulation is expressed. Glucose inhibits sporulation up to seven hours before the appearance of asci (Darland, 1969). Ethanol inhibits up to eight hours before the appearance of asci (Miller, 1971). Kirsop (1954) and Ganesan Holter and Roberts (1958) concluded that commitment occurred at about T_{10}. Transferring the cells to growth medium after this time did not prevent development of asci.

CHANGES IN FINE STRUCTURE DURING ASCOSPOROGENESIS

The literature describing changes that occur in fine structure during ascus formation in Sacch. cerevisiae is extensive. But, despite this profusion of information, the caryology of ascus development has been obscure and the centre of controversy until quite recently. However recent research has clarified many of the detailed events. The processes that lead to the production of four ascospores within an ascus are obviously complex and, in Sacch. cerevisiae, can take at least 48h to complete in appropriate media.

Cytoplasmic changes. The first change which is seen to occur after transfer to sporulation medium is an increase in cell volume. Pontefract and Miller (1962) observed this phenomenon using an optical microscope, but they found that the difference in size between vegetative cells and sporulating cells was not statistically significant. Deysson and Lau (1963) working with Saccharomycodes ludwigii, and Croes (1967a) working with Sacch. cerevisiae, were able

to measure this increase more accurately with the aid of electronic cell-sizing devices. An increase in the size of the nucleus during the first few hours of sporogenesis was noted by Pontefract and Miller (1962) again using an optical microscope.

Small granules become visible in the cytoplasm early in sporulation and these increase in size and number until they obscure the vacuole and nucleus (Pontefract and Miller, 1962; Miller, McClary and Bowers 1963). The granules exhibit ultraviolet-absorptive and-refractive properties. Photomicrographs of these cells at different wavelengths of ultraviolet and visible radiation suggest that several different types of granule are present (Miller et al., 1963). Some of the granules stain very deeply with Sudan Black B and are thought to be lipid (Pontefract and Miller, 1962). These workers noted that the lipid granules increase in size and number as sporulation progresses and are located near to the place where the cytoplasm appeared to be rounding up prior to spore formation. When rudimentary spores had appeared, they observed masses of lipid around the developing spore walls.

An increase in the number of granules, including mitochondria, lipid vesicles and endoplasmic reticulum vesicles, can be seen in electron micrographs of thin sections of developing asci of Sacch. cerevisiae (Hashimoto, Conti and Naylor, 1958, 1959; Hashimoto, Gerhardt, Conti and Naylor, 1960; Mundkur, 1961b; Lynn and Magee, 1970; Moens, 1971; Rapport, 1971) and in electron micrographs of freeze-etched replicas of developing asci of Sacch. cerevisiae (Guth, Hashimoto and Conti, 1972). In contrast to these observations the ribosomes which accumulate in the cytoplasm of vegetative cells rapidly

disappear during the first hours of the sporulation phase (Mundkur, 1961b). Eventually the granules aggregate into the centre of the cell to divide into four rudimentary spores. Some of the granules are included in the spores; others are left in the epiplasm (Miller et al., 1963).

A single large vacuole is present in most cells in the presporulation phase, and this progressively breaks down into several smaller vacuoles (Mundkur, 1961b; Miller et al., 1963; Svihla, Dianko and Schlenk, 1964). The build up of small granules, which has already been mentioned, results in the vacuoles being partially obscured and therefore difficult to observe in the light microscope. In order to overcome this difficulty Svihla et al. (1964) induced sporulation in cells of Sacch. cerevisiae which contained large quantities of S-adenosylmethionine in their vacuoles. This compound strongly absorbs ultraviolet radiation and permits microscopic examination of cytological detail. Cells were grown in a pre-sporulation medium which had been supplemented with methionine, which leads to a greatly increased concentration of S-adenosyl-methionine in the vacuoles (Svihla and Schlenk, 1959, 1960). Vacuolation continues with time until immature spores are visible, after which time the vacuoles break down and their contents are released into the epiplasm. In this study by Svihla et al. (1964) none of the ultraviolet-absorbing material was incorporated into the spores and it eventually disappeared altogether from the ascus.

Meiosis and delimitation of ascospores. Kruis and Satava (1918), Winge (1935) and Winge and Laustsen (1937) provided genetic proof

that ascus formation in yeast involves meiosis. However, descriptions of classical meiosis were based on cytological examination of certain plant and animal cells which are larger than yeast cells. Consequently, cytological studies of meiotic configurations in yeast are impeded by the resolving power of the light microscope. Despite these difficulties, many cytologists have sought to describe the appearance of the yeast nucleus during sporulation in terms of classical meiosis. It has been generally stated, from light-microscope studies of stained cells, that the nucleus divides twice by constriction forming four distinct haploid nuclei (Ganesan, Holter and Roberts, 1958; Pontefract and Miller, 1962). Similar descriptions of nuclear division during sporulation in material fixed for electron microscopy have been made by Hashimoto et al. (1960) and Mundkur (1960b). Lynn and Magee (1970) did not discuss the first division but described an immature ascus containing four separate nuclei which they claimed were the products of the two meiotic divisions.

There is general agreement that the nuclear membrane remains intact during all stages of the meiotic process (Hashimoto et al. 1960; Mundkur, 1960b; Lynn and Magee, 1970; Moens, 1971; Guth et al. 1972). However, in contrast to this observation, Marquardt (1963) asserted that the nuclear membrane does break down during the second division of meiosis.

A novel description of the caryogamy of meiosis was presented by Moens (1971), Rapport (1971) and Moens and Rapport (1971a) who proposed that, during both meiotic divisions, the nucleus does not divide and division of the genetic material occurs within one nuclear

envelope. The haploid nuclei of the spores are formed by nuclear material flowing out of the parent nucleus towards four enveloping spore-wall initials, or 'prospore walls' in Moens' (1971) terminology.

Chromosomes or elements exhibiting chromosomal configurations have not been demonstrated in electron micrographs of thin sections of fixed cells of Sacch. cerevisiae during meiosis (Hashimoto et al., 1960; Mundkur 1961b; Lynn and Magee, 1970; Moens, 1971; Rapport, 1971) or in electron micrographs of freeze-etched replicas of cells of this yeast (Guth et al., 1972). It has, however, been claimed that chromosomes or chromosome-like bodies have been detected during prophase of meiosis in cells of Sacch. cerevisiae stained for light microscopy (McClary, Williams, Lindegren and Ogur, 1957; Pontefract and Miller, 1962). McClary et al. (1957) were able to count the number of bivalents during late prophase and metaphase of a polyploid series of Sacch. cerevisiae and deduced the haploid number to be four.

However the veracity of much of this earlier work is open to question since most workers have failed to discern chromosomes in Sacch. cerevisiae, especially in reports of more recent origin (Croes, 1967a). An interesting case in point is a report by Tamaki (1965) who counted 18 bivalents at metaphase. A squash technique was used, which was made possible by dissolution of the ascan wall by snail-gut enzyme. However it is doubtful whether the bodies observed by Tamaki (1965) really represent chromosomes since, as mentioned earlier, the meiotic yeast cell is packed with small granules some of which may have been mistaken for chromosomes (Matile, Moor and Robinow, 1969).

Engels and Croes (1968) demonstrated the appearance of a synaptinemal complex during meiosis I in electron micrographs of thin sections of Sacch. cerevisiae. These complexes were formed in the interval between synthesis of DNA and the first division. Rapport (1971) and Moens and Rapport (1971b) reported that several synaptonemal complex-like structures, which they termed polycomplexes were formed before meiosis I in the nucleolus. Only one polycomplex formed in each nucleus and, after the first division, it became sequestered in an invagination of the nucleus. It disappeared after the second division.

Division of the nuclear mass is initiated by the appearance of an intranuclear spindle plaque which is contiguous with the nuclear membrane (Moens, 1971; Moens and Rapport, 1971a), or by the appearance of intranuclear spindle fibres (Guth et al., 1972). The spindle plaque, which radiates spindle fibres into the nucleoplasm duplicates and the daughter plaques move along the nuclear membrane to opposite poles of the nucleus where they bracket a short spindle. The spindle elongates and draws the nucleus into a dumbell shape. This represents the reduction division of meiosis, but the nucleus itself does not divide. The spindle disappears, both spindle plaques duplicate and the daughter spindle plaques move apart, again keeping in close proximity with the nuclear membrane until each pair brackets a spindle. This represents the second division of meiosis and again the nucleus remains undivided. Both spindles lengthen, and nuclear material flows in the direction of the four plaques, with the result that the nucleus assumes a lobate appearance. As the lobes expand the main body of the nucleus shrinks. This description of the events which occur during meiosis was first presented by Moens (1971)

and Moens and Rapport (1971a) who used material that had been fixed with glutaraldehyde and stained with osmium tetroxide. They termed this process uninuclear meiosis. Guth et al. (1972), who examined replicas of freeze-etched material, observed a similar sequence of events.

From observations made on material fixed for the electron microscope by permanganate, it is claimed that delimitation of individual ascospores is performed by a single membrane which forms in situ and eventually becomes the spore plasmalemma (Hashimoto et al., 1960; Lynn and Magee, 1970). Spore wall is formed by deposition of material on the outside of this membrane. Marquardt (1963) observed delimitation of ascospores by a double membrane, the inner of which eventually formed the spore plasma membrane and the outer formed the outer spore coat by deposition of material from the epiplasm. It has been reported (Lynn and Magee, 1970) that a second incomplete membrane runs contiguously with the first membrane, spore wall being deposited between the two.

A 'prospore wall', which originates in the cytoplasm behind the spindle plaques, progressively delineates the ascospores in glutaraldehyde-fixed material (Moens, 1971; Moens and Rapport, 1971a). This organelle takes the form of two electron-dense layers bordering an electron-transparent layer. This extends around the nuclear lobe as nuclear material flows toward it and encloses cytoplasm and various cytoplasmic organelles. Eventually the prospore wall completely surrounds the spore, and pinches it off from the parent nucleus. The spore wall develops as part of the prospore wall by deposition of material in the electron-transparent layer. The two

outer layers move apart to accommodate this development.

The permanganate-fixed membrane and the glutaraldehyde-fixed prospore wall are represented by a discrete bilaminar structure, the 'fore-spore wall', in freeze-etched replicas of developing asci (Guth et al., 1972). This structure delimits spores in exactly the same way as the prospore wall. Spore walls develop by accumulation of material between the laminae.

Uninuclear meiosis has not been demonstrated in other ascigerous yeasts or higher ascomycetes, although it has been reported in the Oomycete Saprolegnia terrestris, in which the nucleus assumes a clover-leaf shape during meiosis II (Howard and Moor, 1970). Descriptions of nuclear segregation in yeasts other than Sacch. cerevisiae are scant probably due to the problems associated with fixation and staining of material and to difficulties in interpreting micrographs of thin sections of cells containing several nuclei (Conti and Naylor, 1960a; Bandoni, Bisalputra and Bisalputra, 1967). However Black and Gorman (1971) described a series of events during nuclear segregation in Hansenula wingei which is very similar to uninuclear meiosis. Division of the nucleus starts with an invagination in a plane perpendicular to the first which results in a lobate nucleus. Each lobe of the nucleus becomes detached from the main body of the nucleus, forming four haploid nuclei, before delineation of the spores takes place.

Ascosporogenesis in other Ascomycetes. Delimitation of individual ascospores by double membranes in H. wingei (Black and Gorman, 1971),

in Hansenula anomala (Bandoni et al., 1967) and in Shizosacch. octosporus (Conti and Naylor, 1960b) is very similar to the process already described for Sacch. cerevisiae. In H. wingei and H. anomala the double membranes are derived from fragments of endoplasmic reticulum membranes. Bandoni et al. (1967) suggested that these fragments originate as invaginations of the ascan plasmalemma. The spore wall is derived from material subsequently deposited between the two membranes during spore maturation. The inner membrane becomes the plasmalemma of the ascospore, the outer becomes the outer limiting layer of the spore wall (Bandoni et al., 1967; Black and Gorman, 1971).

In higher Ascomycetes, meiosis is followed by a supernumerary mitotic division producing eight haploid nuclei, the nuclear membrane remaining intact during these divisions. An open-ended cylinder of double membrane, termed the 'ascus vesicle' (Carroll, 1967), forms around the eight nuclei in all but one of the higher Ascomycetes examined so far. These include Saccobolus kerverni (Carroll, 1967), Pyronema domesticum (Reeves, 1967), Hypoxylon fragiforme(Greenhalgh and Evans, 1968), Podospora anserina (Beckett, Barton and Wilson, 1968; Zickler, 1970) and Ascobolus viridulus (Oso, 1969). Individual ascospores are distinguished from one another and the surrounding epiplasm by progressive constriction of the ascus vesicle around the individual nuclei. The primary wall of the ascospore is formed between the two membranes of the ascus vesicle which move apart to accommodate it, the inner membrane becoming the plasma membrane of the spore.

Carroll (1967) and Zickler (1970) reported that the ascus vesicle is

derived from flattened membraneous vesicles which are visible near the periphery of the ascus. The vesicles unwind and fuse together into a continuous system of double membranes. The vesicles arise from invaginations of the nuclear membrane, which fuse with fragments of endoplasmic reticulum membranes forming closed vesicles bounded by a pair of concentric double membranes. Syrop and Beckett(1972) have shown that the spore-delimiting membranes of Taphrina deformans are derived by invagination of the plasma membrane of the ascus at specific sites adjacent to the spore nuclei. Spores are delineated immediately by these membranes without the intermediary step of forming an ascus vesicle.

It can be appreciated that in all species of Ascomycetes from the asciferous yeasts to higher forms, demarcation of ascospores is mediated by a system of double membranes, between which the spore wall develops. In Sacch. cerevisiae the nucleus remains undivided until the spore nuclei are pinched off by the spore delimiting membranes; in higher ascomycetes division of the nucleus, without the loss of the nuclear membrane, produces eight nuclei and spores are subsequently delineated by the ascus vesicle.

GENETICS OF SPORULATION

The genetics of meiosis and ascus development in Sacch. cerevisiae are very poorly described in the literature. Analysis of events during ascospore formation in Sacch. cerevisiae and other yeasts has been restricted by several limitations of technique. Firstly, mutants

have to be prepared in haploid culture and then converted to homozygous diploids to allow expression of the defect. Secondly, genetic analysis utilises the products of meiosis; mutants blocked in some stage of meiosis or sporulation are difficult to characterise if meiotic products can not be obtained. Thirdly isolation and selection for the blockage of specific events in the sporulation process is barely possible since very few sporulation-specific events are known.

Recently several technical innovations and improvements have made progress in overcoming some of these restrictions. Bresch, Muller and Egel (1968) using Schizosaccharomyces pombe, were able to circumvent the first limitation by employing a homothallic strain which allowed the expression of recessive alleles. The particular homothallic strain they employed was capable of mating with either of the heterothallic strains and this permitted isolation of mutants able to sporulate with both heterothallic strains. In this way 300 mutants of meiosis or sporulation were isolated and, by complementation tests and linkage studies, the mutations were shown to be within 24 genes, five necessary for meiosis, 18 necessary for maturation of the spores and one necessary for the dissolution of the separating wall during conjugation.

Esposito and Esposito (1969) isolated 75 mutants of Sacch. cerevisiae which were unable to form asci. These were allotted to various classes; 10 are asporogenous at all temperatures, 16 are sensitive at 20°C and 49 are sensitive at 34°C. The mutants, unable to sporulate at 34°C, but capable of sporulating at 25°C, were examined in detail. Genetic analysis of these mutants was carried out at the

permissive temperature (25°C) but investigations into the effect of the mutations were carried out at the restrictive temperature (34°C).

The mutations were produced in a strain of Sacch. cerevisiae which carries two diploidising genes. These cause spontaneous conversion of either the a or α locus to the opposite type. Haploid ascospores are then able to fuse with each other giving rise to homoallelic diploids. Complementation tests with these mutants revealed eleven separate cistrons and enabled the number of genes specifically involved in meiotic and sporulation function to be estimated at 48 ± 27 loci (Esposito, Frink, Bernstein and Esposito, 1972).

Esposito and Esposito (1969) looked at the biochemistry of three of these mutants (spo1-1, spo2-1 and spo3-1) in detail. All three mutants start meiosis and continue as far as the first division (i.e. the binucleate stage by Giemsa staining) at the restrictive temperature, but are unable to complete the second division. The DNA content of spo2-1 doubled as did the DNA content of the wild type. The DNA content of spo1-1 and spo3-1 increased but only slightly. RNA and protein synthesis and turnover in the three mutants were normal, and all three mutants lost the ability to form colonies (i.e. they became 'committed' to sporulation) after exposure to sporulation medium. Esposito et al. (1969) suggest alternative interpretations. Either commitment to meiosis has taken place even though the cells are unable to complete the process or, pleiotropic effects of the spo mutation cause loss of viability. If the former interpretation is correct then commitment may precede the start of the meiotic division. If this is so the processes which involve commitment are as yet unknown.

In Sacch. cerevisiae sporulation is controlled by the mating-type alleles a and α; diploids which are heterozygous at this locus can sporulate whereas homozygous diploids fail to sporulate (Roman and Sands, 1953). Roth and Lusnak (1970) reported that strains homozygous with respect to the mating-type alleles, when incubated in sporulation medium, fail to synthetise DNA or to show recombination between markers. Now, cells which had completed DNA replication immediately before being transferred to sporulation medium should have enough DNA to undergo meiosis without any further duplication of DNA. The absence of DNA synthesis, recombination and sporulation suggest that the mating-type alleles control an event early in sporulation. An event which need not necessarily be duplication of DNA.

Although a prerequisite for meiosis is heterozygosity of the mating-type alleles, the strain need not be a true diploid. Roth and Fogel (1971) have shown that haploid strains which are aneuploid (n + 1) solely for the chromosome bearing the mating-type locus are able to undergo meiosis, although assembly of ascospores does not proceed to completion. In order to detect recombination a pair of leucine heteroalleles (e.g. $\underline{leu}_{2-1}/\underline{leu}_{2-2}$) were included on the disomic chromosome. Roth and Fogel (1971) were able to isolate 91 mutants of this aneuploid which showed diminished or no meiotic recombination. Recombination is thought to entail a sequence of events that includes breakage of DNA strands, formation of DNA heteroduplex regions, DNA degradation and synthesis and rejoining of ends. Mutants of recombination might be expected to represent lesions in genes controlling endonucleases, polymerases and ligases.

CHANGES IN COMPOSITION WHICH ACCOMPANY ASCOSPOROGENESIS

The convention of Croes (1967a) is adopted in the text for indicating the passage of time during sporulation; T_0 is the time at which the sporulation medium is inoculated with cells and T_n is the time n hours later.

Sporulation is accompanied by an increase in the dry weight of the developing ascus. Croes (1967a) found an increase of 46% over the value at T_0, whereas Roth (1970) found an increase of 90%. Esposito, Esposito Arnaud and Halvorson (1969) reported that the dry weight increased by 60% at T_{12} and declined thereafter to an increase of 50% at T_{50}. The number of cells in the medium during sporulation does not increase. Consequently quantitative data obtained during the course of sporulation can be expressed in two ways; firstly as a fraction of unit dry weight or secondly as a fraction of unit cell number. Most contemporary workers, employ the latter method of expressing results, and this method will be adopted in the text unless specifically stated otherwise.

The changes that occur in the cell volume during sporulation were discussed in the first section of this Introduction. The changes that occur in individual cellular fractions are discussed in the later sections.

Nucleic acids In the germ cells of higher plants meiosis is preceeded by duplication of the genome and hence a doubling of the DNA content. Sherman and Roman (1963) followed replication of DNA during sporulation in Sacch. cerevisiae in the presence of tritiated thymidine, and found

that incorporation of thymidine ceased after T_{12}. Not all of the label was found in thymine so that they were unable to observe the kinetics of DNA synthesis. Direct estimations of DNA extracted from sporulating cells show that the DNA content doubles between T_3 and T_{15} (Croes, 1967a; Esposito et al., 1969; Roth and Lusnak, 1970; Sando and Miyake, 1971). Croes (1967a) observed the microscopic appearance of cells at these times, and concluded that the meiocytes had completed part of prophase of meiosis I before synthesis of DNA could be detected.

The number of ribosomes seen in the cytoplasm decreases quite markedly at the onset of sporogenesis (Mundkur, 1961b) and it is to be expected that the content of RNA reflects this decrease. However, conflicting results have been published. Croes (1967a) reported that the RNA content of sporulating cells gradually declines, but Esposito et al. (1969) reported that the RNA content had increased by 50% at T_{10}, although at T_{50} this had fallen slightly to give an overall increase of 20%. Kadowaki and Halvorson (1971a) published slightly different values; RNA increased by 25% at T_{10} but fell to the original value by T_{42}.

Pulse labelling sporulating cells with radioactive adenine, phosphate, or uracil reveals that synthesis of RNA proceeds continuously during the sporulating cycle with two peaks of maximum synthesis, one at T_{10} and the second between T_{20} and T_{25} (Esposito et al., 1970). These findings suggest significant RNA turnover in sporulating cells.

During sporogenesis, a new species of ribosomal RNA, a 20S RNA, has been identified (Kadowaki and Halvorson, 1971a). It appears during the period T_0 to T_{12} and is synthetised preferentially in comparison with 18S RNA and 26S RNA. The function of this new RNA is as yet unknown. Hybridisation

experiments using nuclear and mitochondrial DNA suggest that 20S RNA originates in the nucleus (Kadowaki and Halvorson, 1971b) and it is suggested that this species of RNA is a high molecular-weight precursor of cytoplasmic RNA.

Abdel-Wahab, Miller, Gabriel and Hoffmann-Ostenhof (1961) reported that the pool of free ribonucleotides in mature asci was very much smaller than in vegetative cells. The effect was most noticeable in the nucleoside di- and tri-phosphate fractions and in a sugar derivative of uridine diphosphate fraction. The authors suggested that the sugar-UDP fraction is used up during spore-wall synthesis. Sando and Miyake (1971) reported a similar decline in the pool of deoxynucleotides and deoxynucleosides.

Amino acids and proteins According to Croes (1967a) the changes that occur in protein content during the early stages of sporulation are complex. From T_0 to T_2 there is a net synthesis of about 10%; after T_4, which coincides with the start of DNA synthesis, there is a slight fall in protein content. The level is then maintained up to T_{12}, after which time the protein content declines steadily. Esposito et al. (1969) found there was a net synthesis of protein of about 30% from T_0 to T_{10}. This level was not maintained and the level declined to the original value by T_{40}. Kadowaki and Halvorson (1971a) and Sando and Miyake (1971) reported that a net protein synthesis of about 10% from T_0 to T_6; this level was maintained up to T_{12} after which time it fell to three-quarters of the original value by T_{42}. The amount of protein in asci after six days in sporulation medium (expressed as a percentage of the dry weight) declined to two-thirds of the value at T_0 (Ramirez and Miller, 1964).

Experiments using radio-actively labelled precursors demonstrated that changes in the content of protein during sporulation are a result of the combined effects of protein turnover and protein synthesis. Protein synthesis is continuous during sporulation and shows two peaks of activity, one at T_6 and one at T_{30} the latter of which coincides with the appearance of mature asci (Esposito et al., 1969; Esposito, Esposito, Arnaud and Halvorson, 1970).

Changes in the size of the pool of free amino acids from T_0 to T_4 were reported by Croes (1967a), and during the whole sporulation phase by Ramirez and Miller (1964) who again expressed their results in terms of percentage of the dry weight. The individual pools of most amino acids are very much diminished as sporulation proceeds, although the amounts of aspartate, glutamate, arginine and methionine remain virtually unchanged (Croes, 1967a) and free proline, which was not detectable in vegetative cells, is accumulated in relatively large amounts during sporulation (Ramirez and Miller, 1964). Croes (1967a) suggested that net protein synthesis reached a maximum very quickly during sporulation due to a depletion of the amino-acid pool. Since incorporation of ammonia proceeds via glutamate, an amino acid which is not depleted, he inferred that acetate is unable to supply sufficient two-carbon moieties for snythesis of amino acids. The production of amino acids from the tricarboxylic-acid cycle depends on the operation of the glyoxylate pathway, and their depletion suggests that this pathway is insufficiently operative. Inhibition of vegetative growth in sporulation medium is readily overcome by addition of glyoxylate which suggests that sporulation is triggered off by an insufficiency of the glyoxylate cycle (Bettelheim and Gay, 1963).

Ramirez and Miller (1964) reported that, as sporulation proceeds, ammonia

is evolved and amino acids released into the medium. They suggested that proteins were degraded to amino acids which were then either respired endogenously, producing ammonia, or were excreted. Proline accumulated as a result of its failure to pass out of the cells, or by a metabolic deficiency preventing its endogenous respiration.

Carbohydrate Sporulating cells of Sacch. cerevisiae contain considerably more carbohydrate than vegetative cells. Pazonyi and Markùs (1955) found that developing asci contain up to 30% more glucan and mannan, 15% more trehalose, but the same amount of glycogen. However it was later claimed, on the basis of staining with Lugol's iodine, that there is an increase in the content of glycogen during the early phases of sporulation (Pontefract and Miller, 1962).

Roth (1970) reported that 67% of the dry weight increase recorded during sporulation is due to synthesis of carbohydrate which consists of trehalose and trichloroacetic acid-insoluble components. Most of the extra carbohydrate was synthetised during the phase preceeding the appearance of ascospores; the content of trehalose increased 18-fold and this disaccharide was eventually localized in the spores and trichloroacetic acid-insoluble carbohydrates increased in amount by a factor of 3.6. The accumulation of trehalose during sporulation is in agreement with previous observations that trehalose is only synthetised from a suitable carbon substrate in the absence of an assimilable nitrogen source, or when cell division is otherwise inhibited (Roth, 1970).

Lipids

This section of the Introduction includes a discussion of changes in lipid composition during ascosporogenesis, and also of effects of growth conditions on yeast lipids, and a description of the various classes of lipid which are synthetised by yeast. These topics reflect the main content of the investigations described in this thesis, namely the lipid changes during ascosporogenesis.

The number of fat globules, identified by their affinity for Sudan Black B, in the cytoplasm of cells increases within five hours in sporulation medium (Pontefract and Miller, 1962). The amount of visible fat was seen to increase until the time spores walls were developing; once the spores had matured the fat content was very much reduced.

Most of the published electron micrographs of thin sections through sporulating cells show lipid vesicles present in the cytoplasm (Hashimoto et al., 1958, 1960; Lynn and Magee, 1970; Moens, 1971; Rapport, 1971; Guth et al., 1972). The number and size of these granules increase until spores begin to appear; after that time some are incorporated into the spore cytoplasm whilst many disappear.

Chassang, Roger, Vezinhet and Galzy (1972) found that the amount of lipid extracted by an azeotropic mixture of benzene, ethanol and water from disintegrated cells and developing asci increased as sporulation progressed. Two classes of lipid were analysed, namely saponifiable lipids (total fatty acids) and unsaponifiable lipids (total sterols). About 90% of presporulation cells harvested in the mid-exponential phase of growth, and only 40% from the stationary phase of growth form asci. The increase in size of the total sterol fraction is much greater in asci derived from

mid-exponential cells than in asci derived from stationary-phase cells.

The kinetics of incorporation of acetate into lipids have been studied by Esposito et al. (1969) and Miyake, Sando and Sato (1971). Esposito and his colleagues found that incorporation of [2 - ^{14}C] acetate proceeds in two stages, the first stage being completed by T_{10}, while the second stage occurs from T_{22} to T_{30}. Miyake and his colleagues reported that incorporation of [1 - ^{14}C] acetate was most rapid from T_0 to T_8 and slowed down toward T_{30}; even so in this period (T_8 to T_{30}) incorporation of isotope into the lipid fraction proceeded at a much faster rate than into other fractions during the same period.

Effect of growth conditions on yeast lipids Growth conditions which cause alterations in the lipid content or lipid composition in yeast include: composition of the growth medium, growth cycle, growth rate, growth temperature and oxygen tension. The effect of growth rate has been studied using chemostat-grown cells. Lowering the growth rate leads to increased synthesis of sterol esters while the contents of sterol and triacylglycerol remain unchanged and a decreased synthesis of unsaturated fatty acids and phospholipids (Hunter and Rose, 1972). The main influence of temperature is to increase the synthesis of lipids containing unsaturated acyl residues as the growth temperature is lowered below the optimum value (Farrell and Rose, 1967). However other reports of growth temperature effects in yeast lipids are conflicting; Kates and Baxter (1962) reported an increase in unsaturated fatty acids when the growth temperature of Candida utilis was lowered, whereas MacMurrough and Rose (1971) found only minor alterations in fatty acids as the growth temperature was decreased. Hunter and Rose (1972) reported that growth temperature of both

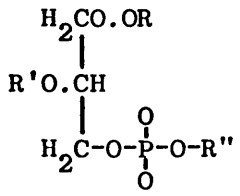
batch grown cells and chemostat-grown cells had very little effect on the fatty-acid composition of Sacch. cerevisiae. However, when the growth temperature is lowered, there is an increase in the content of total lipid, total fatty acids, phospholipids and triacylglycerols. Cells of Sacch. cerevisiae synthetise a greater proportion of unsaturated fatty acids when grown under anaerobic conditions (Jollow, Kellerman and Linnane, 1968). These workers also found that lowering the oxygen tension of the growth medium lowered the contents of all classes of lipid except for squalene, which increased in content. Sacch. cerevisiae becomes auxotrophic for sterols (Andreasen and Stier, 1953) and for fatty acids (Andreasen and Stier, 1954) when it is grown under completely anaerobic conditions.

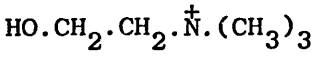
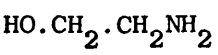
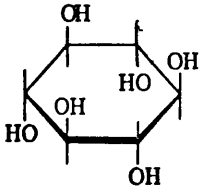
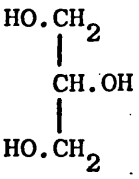
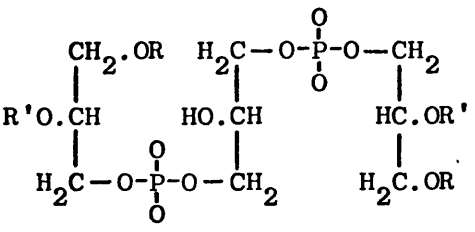
Biochemistry of Yeast lipids The biochemistry of yeast lipids is perhaps harder to deal with than that of other major constituents. Lipids are much more heterogenous than proteins, carbohydrates or nucleic acids. In this thesis lipids are defined as those compounds which are soluble in organic solvents and which are virtually insoluble in water.

i) Phospholipids Table 1 is a list of substituted diesters of sn-glycero-3-phosphoric acid which have been detected in yeast (Hunter and Rose, 1971). It must be realised that each name represents a class of lipid, individual members of each class differ by the nature of the acyl residues and their positional distribution.

Table 1. Phospholipids found in yeast.

Glycerol phosphate derivatives



Phosphatidylcholine (lecithins)	R saturated fatty acid	
	R' unsaturated fatty acid	
	R" derived from choline	
Phosphatidylethanolamine (cephalins)	R and R' as for lecithins	
	R" derived from ethanolamine	
Phosphatidylserine	R and R' as for lecithins	
	R" derived from serine	
Phosphatidylinositol	R and R' fatty acids	
	R" derived from myo-inositol	
Phosphatidylglycerol	R and R' fatty acids	
	R" derived from glycerol	
Diphosphatidylglycerol		
Phosphatidic acid	R and R' fatty acids	
	R" H or	

ii) Acylglycerols The acyl residues of yeast triacylglycerols range from C_8 to C_{24} and include saturated and unsaturated representatives, in particular $C_{16:1}$ and $C_{18:1}$ acids (Kates and Baxter, 1962).

Diacylglycerols and monoacylglycerols, which are diesters and monoesters respectively of glycerol and fatty acids, have also been detected in extracts from yeast (Kates and Baxter, 1962). Diacylglycerols of yeast are mainly 1,2,diacyl-sn-glycerols, although they include small amounts of 1,3,diacyl-sn-glycerols.

iii) Fatty acids The vast majority of fatty-acids in yeast lipids are straight-chain saturated or unsaturated, even-numbered carbon (C_{14}, C_{16} and C_{18}) acids. Other types of fatty acids, including branched chain and odd-numered carbon acids have been detected in yeasts (Kates and Baxter, 1962), but invariably in small amounts.

iv) Sterols The commonest yeast sterols are ergosterol, zymosterol (Dulaney, Stapley and Simpf, 1954) and $\Delta^{5,7,22,24(28)}$ergostatetraene-3β-ol (Breivik et al., 1954; Longely, Rose and Knights, 1968). Minor qantities of other sterols have been reported to be present in yeast, these include ascosterol and fecosterol (Weiland, Rath and Hess, 1941) and episterol (Weiland and Gough, 1930). Sterols occur in yeast in both the free form and esterified with long chain fatty acids (Smedley-MacClean and Thomas, 1920).

METABOLIC CHANGES ASSOCIATED WITH ASCOSPOROGENESIS

Previous sections of this Introduction described in detail the changes in fine structure and the changes in composition that occur during ascus formation in Sacch. cerevisiae. It is evident that the yeast cell must undergo a striking reorganisation of its metabolic activity to effect these morphological and biochemical changes. In sporulation medium acetate is the prime source of energy; hence the rate of acetate consumption can be held to be indicative of the general metabolic state of the cell. Cells very rapidly adapt to sporulation medium and oxidation of acetate proceeds at the maximum rate after T_2 (Miller, Hoffmann-Ostenhof, Schieber and Gabriel, 1959; Croes, 1967a). Evolution of carbon dioxide proceeds without any initial lag in sporulation medium and continues in a linear fashion up to T_{20} (Miller et al., 1959; Esposito et. al., 1969). These results indicate that there is a speeding up of aerobic respiration in sporulation medium and that cells are able to respire acetate almost immediately.

Croes (1967b) has put forward a teleological theory that meiosis is induced by a series of events which start in the presporulation medium. The first event which Croes sees of importance is the fermentation-to-respiration change, which marks the end of the log phase of growth, and is brought about by the disappearance of glucose from the medium. During the later stages of the presporulation phase the cells metabolise ethanol, the by-product of the glucose fermentation, which is metabolised via acetate and the tricarboxylic acid cycle. Consequently the presporulation cells are competent to metabolise acetate. High concentrations of RNA and protein are built up during the log phase. However, once the fermentation-to-respiration change has taken place the cells fail to maintain these levels and they become

progressively unable to undergo sporogenesis.

Mandlestam (1971) has suggested that the changes which occur at the end of log phase as a response to starvation conditions are observed in many different cellular systems, including E. coli, Gram-positive bacilli, slime molds and Acetabularia. During the log phase of growth maximum cell yields are achieved only if all unnecessary enzyme systems are severely repressed. At the beginning of the stationary phase, or during periods of starvation, degradation of protein and ribosomal RNA occur. These processes get rid of any unwanted enzymes which were present during logarithmic growth and provide a plentiful supply of amino acids for the rapid synthesis of new enzyme systems as they are required.

Very little is known about the activities of various enzyme systems during ascus formation although it is assumed that extensive changes in enzyme composition and activity must occur. Chen and Miller (1968) have shown that the ability of sporulating cells to degrade haemoglobin increases to a maximum after T_{17}. This time coincides with the stage just before the appearance of spore walls. The increase in proteolytic activity would account for the high rate of protein turnover observed by Esposito et al. (1969, 1970) and the decline in total protein observed by Croes (1967a) and Esposito et al. (1969). Cells which are deficient in the ability to degrade protein in sporulation medium are also asporogenic (Chen and Miller, 1968).

During sporulation synthesis of some enzymes will be repressed. Miller and Hoffman-Ostenhof (1964) have suggested that enzymes which catalyse reactions of aerobic glycolysis will be repressed. On the other hand many enzyme systems will be induced or enhanced. Glucose, which is known to

inhibit induced enzyme formation, especially those of the TCA cycle, also strongly inhibits sporulation. Oxygen promoted enzyme synthesis and is also required for sporulation.

Activities of TCA cycle enzymes, including aconitase, NADP-linked isocitrate dehydrogenase and succinate dehydrogenase all increase slightly during sporulation (Miyake et al., 1971). However, the activities of these enzymes increased by the same amount in an asporogenic strain when incubated for the same period in sporulation medium. However the activity of isocitrate lyase, which is the first enzyme of the glyoxylate bypass, increased only in the sporogenic strain. This supposed activity of the glyoxylate bypass is supported by two observations. Firstly, carbon dioxide evolved during sporogenesis is derived from the carboxyl group of labelled acetate (Miyake et al., 1971) and secondly, addition of glyoxylate to acetate sporulation medium accelerated yeast sporulation in the absence of carbon dioxide (Bettelheim and Gay, 1963).

Croes (1967a) provided evidence that induction of enzymes occurs in the early stages of sporulation. When ethionine is added to sporulating cells, meiotic DNA duplication and maturation of the spores are inhibited, but protein and RNA patterns are essentially unaltered. The action of ethionine can be arrested by addition of methionine. Both processes were only blocked when ethionine was present some time before they occured. Meiosis, which occurs from T_3 to T_{15}, was blocked when ethionine was present from T_0 to T_2; maturation of spores, which occurs from T_{12} to T_{18}, was blocked by the presence of ethionine from T_0 to T_7. If ethionine is present during the last two hours of the presporulation phase, sporogenesis is severely inhibited. These results suggest that ethionine is preventing some cellular processes occuring, probably induction of enzymes, which are

required later on in the sporulation cycle.

The hypothesis that induction of enzymes is necessary for ascus formation is further corroborated by the observation that the frequency of asci is very much diminished when protein synthesis is prevented by the presence of actidione at any time before the completion of ascus development (Esposito et al., 1969).

In conclusion little is known of the changes in the lipid content of yeast during ascosporogenesis. The purpose of the investigations described in this thesis was to carry out a detailed examination of the lipid composition of cells of Saccharomyces cerevisiae during ascosporogenesis and to correlate these results with the modifications in fine structure which occur during this process.

METHODS AND MATERIALS

METHODS

Organism The yeast used in this study was a strain of Sacch. cerevisiae DCL 740 kindly provided by R.R. Fowell. It was maintained on slopes of malt wort-agar as described by Dixon and Rose (1964). Stock cultures were stored at 4°C. This strain produces a high proportion (80-90%) of four spored asci. When the sporulating ability of the yeast declined, single-cell isolates were obtained by plating on malt wort-agar medium. The majority of these isolates produced a high proportion of asci.

Growth of cells Cells were grown in a presporulation medium containing 1.3% (w/v) nutrient broth (Oxoid), 5% (w/v) glucose and 1% (w/v) yeast extract (Oxoid); pH 6.3 (Fowell, 1969). Portions (1 litre) of medium were dispensed into 2 litre round flat-bottomed flasks which were plugged with cotton wool, and sterilized at 115°C for 15 min. Batches of medium were inoculated with a loopful of cells from a slope culture, and were incubated at 30°C as described by Patching and Rose (1969), except that the magnetic stirrer was rotated at only about 200-300 rotations per min. After 40 h, the culture was in the stationary phase of growth (about 4.75mg dry weight/ml or 1.5 x 10^8 cells/ml). The cells were then harvested by centrifugation at 12,000 g at 3°C, washed twice with water, and resuspended in water.

Production of asci Sporulation of cells was induced by incubating

vegetative cells in a sporulation medium containing 0.5% (w/v) sodium acetate and 1.0% (w/v) KCl (pH 7.0; Fowell, 1969). Batches of sporulation medium (1 litre in a 2-litre round flat-bottomed flask, or 100 ml in a 250 ml conical flask) were inoculated by adding an aqueous suspension of washed vegetative cells to a density of 0.44 mg dry weight (1.44×10^7 cells) per ml. Suspensions were incubated at 25°C, with rapid stirring as described by Patching and Rose (1969) for larger cultures and with orbital shaking for smaller cultures. Production of asci was followed by removing portions of suspension and counting the numbers of 2-, 3- and 4-spored asci using a haemocytometer slide. A cell with an attached bud was counted as one cell. At least 300 cells or asci were counted. Ascospores were visible in asci after about 24 h incubation, and spore formation was complete after about 48 h. Nevertheless, the suspensions were incubated for up to 120 h to allow the spores to ripen. Asci and cells were harvested from the suspension by centrifugation at 12,000 g at 3°C. They were washed twice with water, freeze-dried, and stored at - 20°C in the presence of a desiccant.

Dry weight measurements These were made on cells and asci that had been freeze-dried or dried to constant weight at 80°C under reduced pressure. These methods gave virtually identical values for vegetative cells, but not with developing asci.

Extraction of lipids Lipids were extracted from vegetative cells and asci by a modification of the procedure used by Letters (1968). Portions (500 - 800 mg) of freeze-dried material were extracted with 15 ml ethanol at 80°C for 15 min. The residue was extracted with 3 x 3 ml chloroform-

methanol (1:1, v/v) with stirring, each extraction lasted 2.5h at room temperature (18 - 22°C). At each stage the extracts were filtered through Whatman No. 44 paper. The ethanol extract and the three cloroform-methanol extracts were pooled, supplemented with 1 ml chloroform containing butylated hydroxytoluene (0.005%, w/v) as an antioxidant (Neudoerffer and Lea, 1966) and stored at - 20°C. The extract was filtered through a glass sinter (No. 5) and evaporated to dryness in vacuo using a rotary evaporator and a Buchler rotary evapo-mix.

Non-lipid material was removed from the extract by a modification of the procedure of Folch, Lees and Sloane-Stanley (1957). For each 100 mg of material extracted, 1 ml chloroform-methanol (2:1, v/v) was used to dissolve the dry extract and one fifth of this volume of 0.118M KCl was added. The two phases were well mixed and centrifuged at 3,100 g for two min to speed up their separation. The aqueous phase was drawn off and the interface washed three times with 1.5 ml of the aqueous phase of chloroform-methanol-0.118 M KCl (8:4:3, by vol.). The washed lipid extract was evaporated to dryness in vacuo and dissolved in chloroform-methanol (2:1, v/v) and made up to two ml in a graduated flask. Small pans were prepared from aluminium foil, dried overnight in a vacuum desiccator containing silica gel and solid KOH (Rouser, Kritchevsky and Yamamoto, 1967) and weighed; 100 ul of extract was added to each of three pans and the pans returned to the desiccator. The desiccator was evacuated overnight and the pans dried to constant weight.

Analysis of phospholipids Total phospholipid content of the lipid extract was determined by assaying the phosphorus content of a 25 µl portion of the extract using the method of Bartlett (1959) or Chen, Toribara and Warner

(1956). Values for phosphorus content were converted to phospholipid by multiplying by 25 (which assumes an average molecular weight of 800 for a phospholipid). Individual phospholipids in the extract were separated using two-dimensional thin-layer chromatography using plates (20cm x 20cm) coated with a layer (0.25mm) of Kieselgel PF_{254} (Merck). Before use plates were activated by heating at 110°C for 1h and allowed to cool for at least 30 min in the presence of a desiccant. The sample was applied to one corner of the plate and developed in the first direction with chloroform-methanol-ammonia (0.88 sp. gr.) (65:35:5, by vol.) and in the second direction with chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5, by vol.) Individual phospholipids were identified using standard preparations and specific spray reagents (Dittmer and Lester, 1964).

Analysis of neutral lipids Neutral lipids in extracts were separated quantitatively by thin-layer chromatography. Plates (20 x 20cm) with a layer (0.75mm) of Kieselgel $PF_{366+254}$ (Merck) were washed overnight in chloroform. The silica gel was divided into 7 x 2.5cm channels. Two channels were used to run standard lipids, and another two channels were used as blanks in subsequent colorimetric determinations. The sample was applied to the remaining three channels as streaks. Plates were developed with diisopropyl ether-acetic acid (96:4, v/v) to a distance of 9cm above the origin and then in the same direction with petroleum spirit (40-60°C)-diethyl ether-acetic acid (90:10:1, by vol.) to a distance 16cm above the origin. Bands of lipid were located with an ultraviolet lamp, and identified with simultaneously run standards. Quantitative separation of squalene from other neutral lipids was carried out by developing the plates in petroleum spirit (40 - 60°C). Squalene was located by spraying a simultaneously run squalene standard with an ethanolic solution of iodine (2%, w/v in 70%, v/v

ethanol). The silica gel containing each band of lipid was removed from the plate, using a microscope slide, onto a piece of grease-proof paper and transferred to a centrifuge tube.

Phospholipids located at the origin were eluted from the silica gel with 2 x 3ml chloroform-methanol-water (5:5:1, by vol.) followed by 3 ml methanol and 3 ml methanol-acetic acid-water (95:1:5, by vol.). The phospholipid content of the extract was assayed as already described.

Sterols and sterol esters were eluted from the silica gel with 3 x 3 ml portions of chloroform-methanol (4:1, v/v) and assayed by a modification of the Liebermann-Burchard reaction described by Moore and Baumann (1952). Gas-liquid chromatography revealed that the extracts contained only very small amounts of sterols that lack the 5, 7-diene grouping. Consequently, only 5, 7-diene sterols, which are the fast-reacting sterols in the Liebermann-Burchard assay, were determined. Sterol contents were calculated from a standard curve prepared using ergosterol. Sterol esters were dissolved in 0.1 ml benzene and saponified by refluxing for 2h with 0.4ml 10% (w/v) KOH in 90% (v/v) ethanol in a tube fitted with a cold-finger condenser. The sterols liberated were assayed as already described.

Diacylglycerols and triacylglycerols were eluted from the silica gel by 2 x 3ml portions of chloroform followed by 2 x 3ml portions of diethyl ether. The first ether elution was performed in a cold Rotary Evapomix (Buchler Instruments Inc.) without a vacuum for 10 min. Diacylglycerols and triacylglycerols were assayed by a chromotropic acid method (van Handel and Zilversmit, 1957). Contents are expressed as dipalmitin- and tripalmitin- equivalent, respectively, derived from a standard curve prepared using tripalmitin.

Free fatty acids were eluted from the silica gel by 2 x 3ml portions of diethyl ether-methanol (9:1, v/v) followed by 2 x 3ml portions of chloroform. They were assayed by the method of Heinen and de Vries (1966) and contents related to oleic acid-equivalent using a standard curve.

Squalene which was not eluted from the silica gel was assayed by the method of Trappe (1938) which involves bromination of squalene, and iodometric titration of the unreacted bromine.

Sterols were extracted from freeze-dried cells by hydrolysis followed by saponification. Yeast (100mg) was refluxed with 30ml 0.33 N HCl for 1h. The supernatant was extracted with 3 x 30ml diethyl ether, and the residue refluxed with 5ml 7N KOH for 2h at 130°C. The reaction mixture was extracted with 2 x 35ml cyclohexane with shaking. The diethyl ether and cyclohexane extracts were pooled and analysed for total sterol by the Liebermann-Burchard method (Moore and Baumann, 1952) and for ergosterol by the ultraviolet extinction method of Shaw and Jefferies (1953).

Gas-liquid chromatography Samples were analysed using a Pye series 104 model 64 chromatograph, with flame ionization detectors. Sterols were prepared for gas-liquid chromatography by dissolving the lipid extract in 1ml benzene and refluxing with 4ml 1.78M KOH in 90% (v/v) ethanol for 2h. Water (10ml) was added, and the pH value adjusted to 1.0 with HCl. The mixture was then extracted with 3 x 10ml diethyl ether and the extracts dried over sodium sulphate before being concentrated in vacuo. Free sterols were purified by thin-layer chromatography with petroleum spirit - diethyl ether-acetic acid (70:30:2, by vol.) and eluted from the silica gel with 150ml diethyl ether. Trimethylsilyl ethers were prepared by dissolving

the sterols in 1ml dry pyridine, adding 0.2ml hexamethyl disilazane and 0.1ml trimethyl chlorosilane (Sweeley, Bentley, Makita and Wells, 1963). The mixture was shaken and left at room temperature for 15 min. Excess reagents were evaporated off under a stream of nitrogen gas, and the products taken up in chloroform. Alternatively, the reaction mixture was partitioned between petroleum spirit and water, and the petroleum spirit extract concentrated and redissolved in chloroform. Trimethylsilyl ethers of the sterols were separated on 3% OV 17 supported by 100-200 mesh Gas Chrom Q in a 9ft x 1/4 in stainless steel column. The column temperature was 225°C with a nitrogen gas flow rate of 70ml/min; the detector oven temperature was 300°C.

Methyl esters of fatty acids were prepared by refluxing 10-20mg extract in 0.5ml dry benzene with 4ml methanolic HCl (5%, w/v) for 2h. A tube of anhydrous $CaCl_2$ was fitted to the condenser. After adding 10ml water, the methyl esters were extracted with 3 x 10ml diethyl ether. These extracts were dried over $CaCl_2$ before being concentrated. The methyl esters were purified by thin-layer chromatography using petroleum spirit-diethyl ether-acetic acid (90:10:1, by vol.) and were eluted from the silica gel with 150ml petroleum spirit-diethyl ether (1:1, v/v). The eluate was concentrated and redissolved in chloroform before the esters were separated on 15% polyethylene glycol succinate (PEGS) on 85-100 mesh Universal B, or 15% EGSS-X on 100-120 mesh Gas Chrom P. The PEGS was packed in a 5ft x ¼ in glass column and maintained at 155°C; the detector oven was set at 200°C with a nitrogen gas flow of 60ml per min. The EGSS-X was packed in a 5ft x ¼ in stainless steel column, maintained at 175°C with a nitrogen gas flow rate of 50ml/min and the detector oven at 200°C.

The relative amounts of material represented by each peak were calculated by multiplying the peak height by its retention time. This method was found to be more reproducible than the trigonometrical method of multiplying peak height by the peak width at the half height (Pecsok 1959).

Radio-active counting methods Carbon-14 activity in cells and extracts was measured using a Beckman model cpm 200 liquid scintillation spectrometer (Beckman Instruments Limited, Glenrothes, Fife, Scotland). Yeast cells were removed from 5ml suspension containing [U-^{14}C] acetate by filtering through Sartorius or Millipore membrane filters (1.2µm pore size: 2.5 cm diam), washed with 3 x 10ml water, and the cells plus filters dried to constant weight in a vacuum desiccator. They were then placed in a scintillation vial containing 5ml scintillation liquid (toluene: 2-methoxyethanol: 2,5-diphenyloxazole; 3:2:0.003, v/v/w). Bands of silica gel containing various classes of lipid extracted from cells grown in the presence of [U-^{14}C] acetate were scraped off thin-layer plates and transferred to scintillation vials containing 5ml scintillation liquid (toluene: 2,5-diphenyloxazole; 1:0.003, v/w). All samples were counted up to 100 min or to a 2σ statistical counting error usually equal to 1% or 2%. Readings were corrected for average background count using blank areas of silica gel from thin-layer plates.

Measurements of electrophoretic mobility Suspensions of asci and vegetative cells (80mg dry weight/ml) in 50mM sodium acetate buffer were treated with one-third volume snail juice (prepared from the crop of the Roman snail, Helix pomatia; Johnson and Mortimer, 1959) and incubated at

30°C for 24h. The asci and cells were harvested, washed and ascospores released from asci by subjecting a suspension in water at 0°C to sonic treatment for three min with an ultrasonic disintegrator (Measuring and Scientific Instruments Ltd) at 20 kc/s. Release of ascospores from asci was monitored by microscopic examination.

The electrophoretic mobilities of spores were measured in a rectangular closed glass cell (40 x 25 x 1.5mm), thermostatted at 25.0 ± 0.2°C, with the aid of a bench microscope. The latter was equipped with a x40 phase contrast objective and a x10 focussing eyepiece which contained a cross-hatched graticule (Gittens and James, 1960; Somers and Fisher, 1967). Movement was timed over 180 μm in both directions (current reversal) and each mobility value was obtained from at least 20 observations: the standard error of the mean was less than 4%. Electrophoretic measurments were made by using suspensions containing about 10^6 ascospores per ml. Spores were washed twice in the appropriate buffer before suspension in the HCl-NaCl or barbiturate-acetate buffer of the required pH value (Gittens and James, 1963). All buffers had an ionic strength of 0.05.

Electron microscopy Vegetative cells and asci were fixed in 1% (w/v) aqueous potassium permanganate. Preliminary experiments showed that asci could be satisfactorily fixed while suspended in water, but with vegetative cells best results were obtained by first freeze drying the cells and then resuspending them in water prior to chemical fixation. The optimum times for fixation were established with each developmental stage examined, and were as follows: vegetative cells, 4.0h; 6h asci, 5.5h; 12h asci 5.0h; 18h asci, 4.5h; 24h asci, 4.0h. The first ten minutes of fixation in permanganate were carried out under reduced pressure. Fixed material

was washed in water and suspended in liquid 1% (w/v) agar which, after setting, was cut into $1mm^3$ blocks.

These blocks were dehydrated in a graded ethanol:water series, which included 10% (v/v), 20% (v/v), 30% (v/v), 50% (v/v), 70% (v/v), 90% (v/v), followed by two changes of absolute ethanol; each treatment lasted 10 min. The blocks were stained for one hour during dehydration in 2% (w/v) uranyl acetate in 70% (v/v) ethanol. Residual water was removed from the blocks by two 10 min soakings in propylene oxide. The blocks were infiltrated with epon by passage through graded epon: propylene oxide mixtures made up of respectively 1:3 (v/v), 1:1 (v/v) and 3:1 (v/v). Each treatment was carried out in a covered watch glass and lasted 24 h. Infiltration with epon was completed by soaking the blocks in three changes of epon. At the beginning of each change the preparation was held at reduced pressure for 15 min. Each stage was carried out in an open watch glass and lasted 12h. The blocks were finally embedded in epon at a temperature of $45^{o}C$, held under reduced pressure for 15 min and then polymerized at $60^{o}C$ for 48h. Sections were cut with a diamond knife on an LKB ultramicrotome, stained in lead citrate (Reynolds, 1963) and viewed with an AEI EM 6M electron microscope. Photographs were taken on Dupont Graphic Arts film, processed with Kodak DG 10 high contrast developer and printed on Ilford paper.

Optical Microscopy Vegetative cells and asci were suspended in culture medium on a microscope slide, covered with a coverslip, blotted dry and sealed with paraffin wax. Slides were examined in a Leitz Orthoplan microscope using phase-contrast and darkfield illumination. Photographs were taken with a Leitz Orthomat camera on Kodak Ektachrome B film and printed on Kodak paper.

Materials

Chemicals were supplied by the following manufacturers or agents:-

Field Instruments Limited, Richmond-upon-Thames, Surrey, England (agents for Applied Science Laboratories Incorporated) supplied standard mixtures of fatty acid methyl esters and 15% EGSS-X on Gas Chrom P.

Sigma Chemical Company Limited, Lettice Street, London, supplied standard lipids.

Phase Separations Limited, Queensferry, Flintshire, Wales, supplied 15% PEGS on Universal B and 3% OV-17 on Gas Chrom Q.

Anderman and Company Limited, Battlebridge House, Tooley Street, London (agents for E. Merck A.G.) supplied silica gels.

The Radiochemical Centre, Amersham, Bucks, England supplied the sodium salt of [U-^{14}C] acetic acid.

L'Industrie Biologique Francaise, SA., Gennevilliers, Seine, France supplied snail-gut juice, the digestive juice of Helix pomatia.

All other chemicals were analytical grade (i.e. 'AnalaR' or 'AR') or of the highest purity available commercially. Chloroform and methanol were redistilled before use. Pyridine was refluxed over barium oxide for 2h, redistilled and then stored over potassium hydroxide. Benzene was stored and dried over sodium wire. Methanolic-HCl was prepared by bubbling HCl gas through previously dried HCl (Lund and Bjerrum, 1931) and the increase in weight measured. It was then diluted to 5% (w/v) with dry methanol.

RESULTS

CHANGES IN FINE STRUCTURE DURING ASCUS DEVELOPMENT

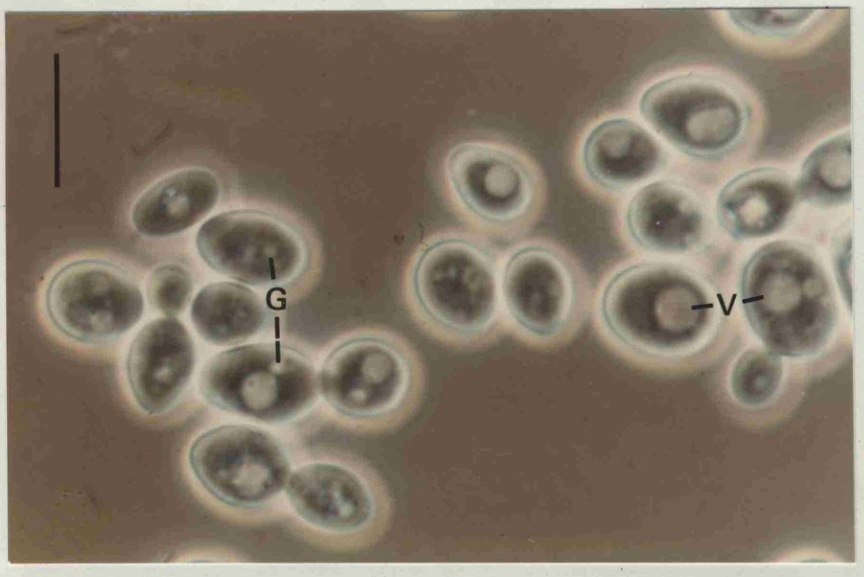
Cells and developing asci were examined as unstained, wet preparations by phase-contrast and darkfield illumination in an optical microscope, or examined in an electron microscope.

Optical microscopy. Cells from the presporulation culture (Plate 1) usually contain a large prominent vacuole and occasionally smaller subsidiary vacuoles. Small granules are visible in the cytoplasm. With darkfield illumination these granules show as pinpricks of light; with phase-contrast illumination they show both as light and dark spots. Active Brownian movement made photography of these granules difficult.

After 12h incubation in sporulation medium (Plate 2), there is an increase in the number of granules, some of which appear to cluster around the vacuole. The process of granulation continues during the next 6h (Plate 3) with the result that many cells appeared to be packed with granules. In cells examined by phase-contrast the vacuoles are obscured by the granules, but in cells examined by darkfield illumination the vacuoles appeared as blank areas surrounded by a mass of granules.

At T_{24} spores are just visible in some cells, (Plate 4). These spores, like the cytoplasm of the ascus prior to their appearance, are highly granulated. By T_{48} outlines of the spores are much more distinct, and the contents of both spores and epiplasm are much less granular, (Plate 5).

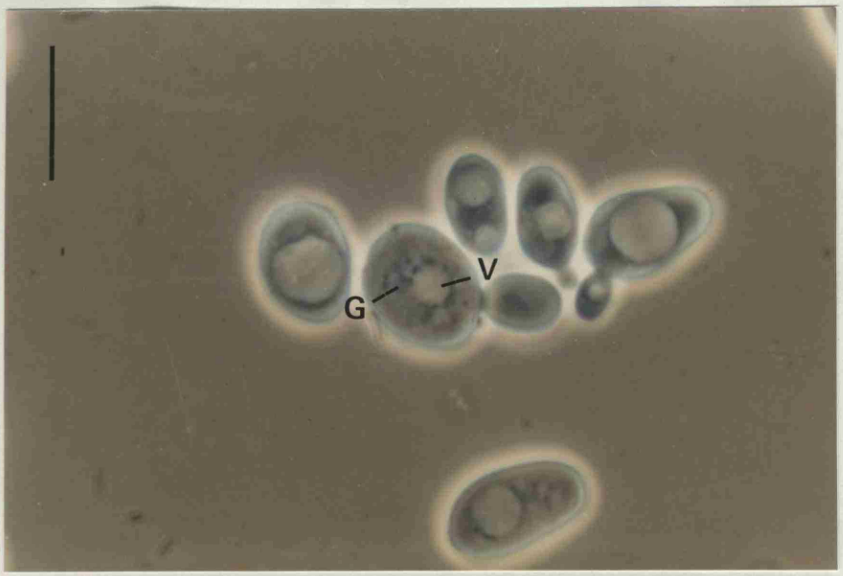
Plate 1.



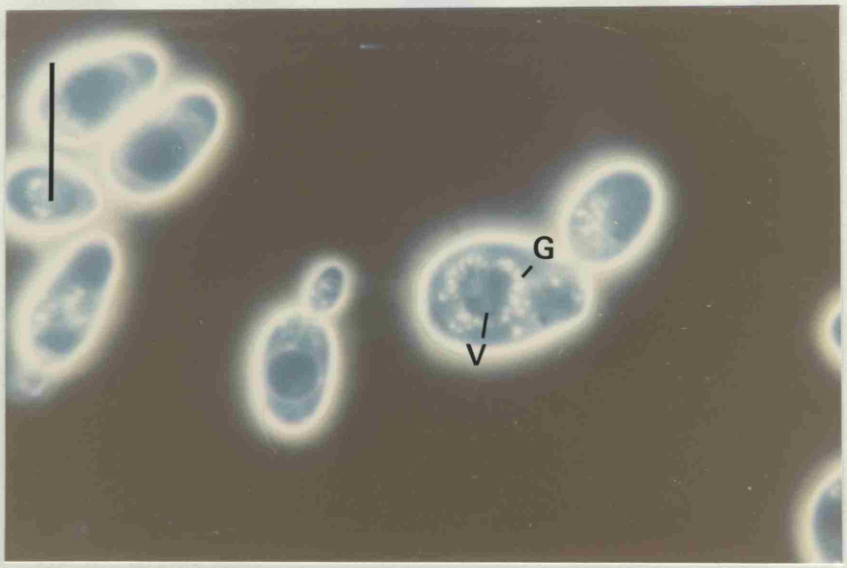
Vegetative cells of Saccharomyces cerevisiae, by phase-contrast illumination. Most cells contain a prominent vacuole (V) and several refractile granules (G). The bar represents 10μm.

Vegetative cells of Saccharomyces cerevisiae by dark-field illumination. The vacuole is less prominently displayed, but refractile granules (G) are present. The bar represents 10μm.

Plate 2.

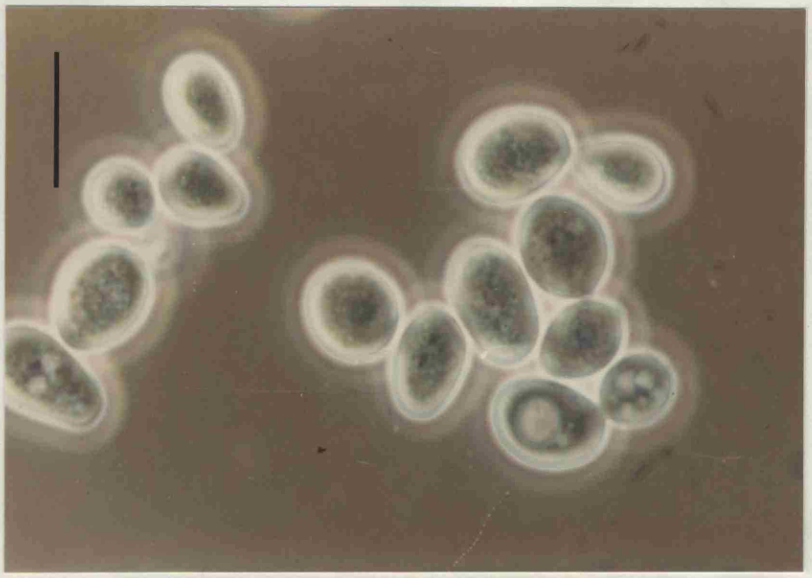


Developing asci, after 12h incubation in sporulation medium, phase-contrast illumination. Prominent vacuole (V) is seen to be surrounded by dark granules (G). The bar represents 10μm.



Developing asci after 12h incubation in sporulation medium, dark-field illumination. Vacuole (V) is surrounded by refractile granules (G). The bar represents 10μm.

Plate 3.



Developing asci after 18h incubation in sporulation medium, by phase-contrast illumination. The vacuole has either disappeared or is obscured by the numerous small granules which pack the cell. The bar represents 10μm.

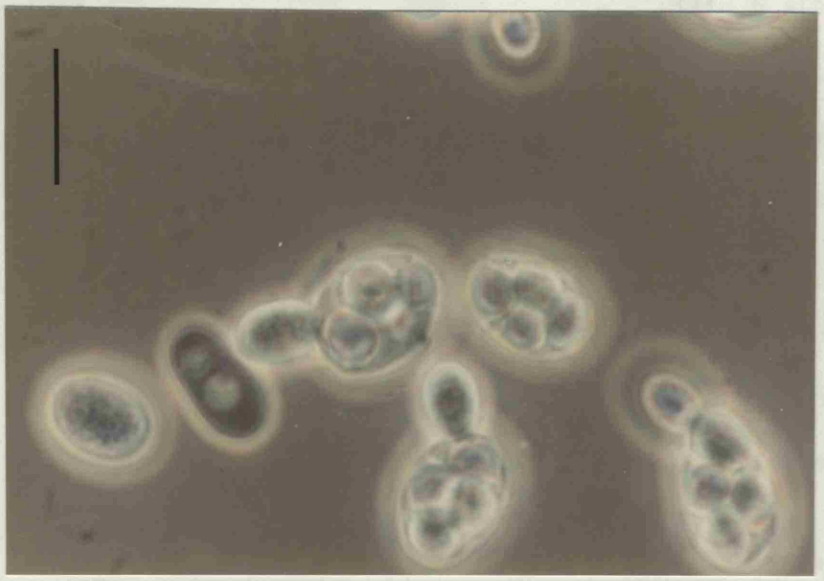
Developing asci after 18h incubation in sporulation medium, by dark-field illumination. Each cell is full of refractile granules except for the central region, possibly the nucleus, which remains relatively clear. The bar represents 10μm.

Plate 4.

Asci after 24h incubation in sporulation medium, phase-contrast illumination. Immature ascospores (IAS) are just visible in some cells. The bar represents 10μm.

Asci after 24h incubation in sporulation medium, dark-field illumination. Immature ascospores (IAS) are visible in some cells. The bar represents 10μm.

Plate 5.



Asci after 48h incubation in sporulation medium, phase-contrast illumination. Mature ascospores are clearly visible in many asci. The bar represents 10μm.

Asci after 48h incubation in sporulation medium, dark-field illumination. Mature ascospores are clearly visible.
The bar represents 10μm.

Electron microscopy. Vegetative cells, examined immediately before inoculation into sporulation medium, contain a more or less spherical nucleus and a centrally placed vacuole. Plate 6 shows a typical example. In this section, as far as the vacuole is concerned, only the tonoplast is visible. A few mitochondria and membrane profiles of endoplasmic reticulum are visible near the periphery of the cell.

Few changes occur in the fine structure of developing asci from T_0 to T_{12}. There is an increase in the number of mitochondria and an increase in the amount of endoplasmic reticulum membranes. It is worthy of note that these organelles remain aligned around the periphery of the cell. The appearance of the nucleus hardly changes during this period. The vacuole hardly changes during this period. The vacuole becomes fragmented during the period T_6 to T_{12} (Plates 6, 7, 8).

Incubation for a further 6h hours leads to more extensive changes; Plate 9 is representative of asci at T_{18}. Numerous randomly situated electron-transparent vesicles, which probably contain lipid, appear in the cytoplasm. Each vesicle is seen to be surrounded by an electron-dense layer, which could be interpreted as a boundary membrane. However at high magnification (see plates 15, 16) the typical unit membrane is not found in cells examined at T_{24} (Plates 10, 11, 12, 13, 14, 15, 16, 17, 18). During this period the nucleus becomes very irregular in outline, possibly reflecting a state of division (compare Plates 10 and 11). Plate 11 shows a cell whose nucleus has assumed a multilobate shape which possibly marks the end of the meiotic division. Each lobe of the nucleus is partially enveloped by two parallel membranes, the spore delimiting membranes. A close-up

Plate 6. Vegetative cells of Saccharomyces cerevisiae. The nucleus (N) and the tonoplast of the tangentially sectioned vacuole (V) occupy the central region of the cell. A few mitochondria (M) and membrane profiles of endoplasmic reticulum (ER) are visible around the periphery of the cell. The bar represents 1µm.

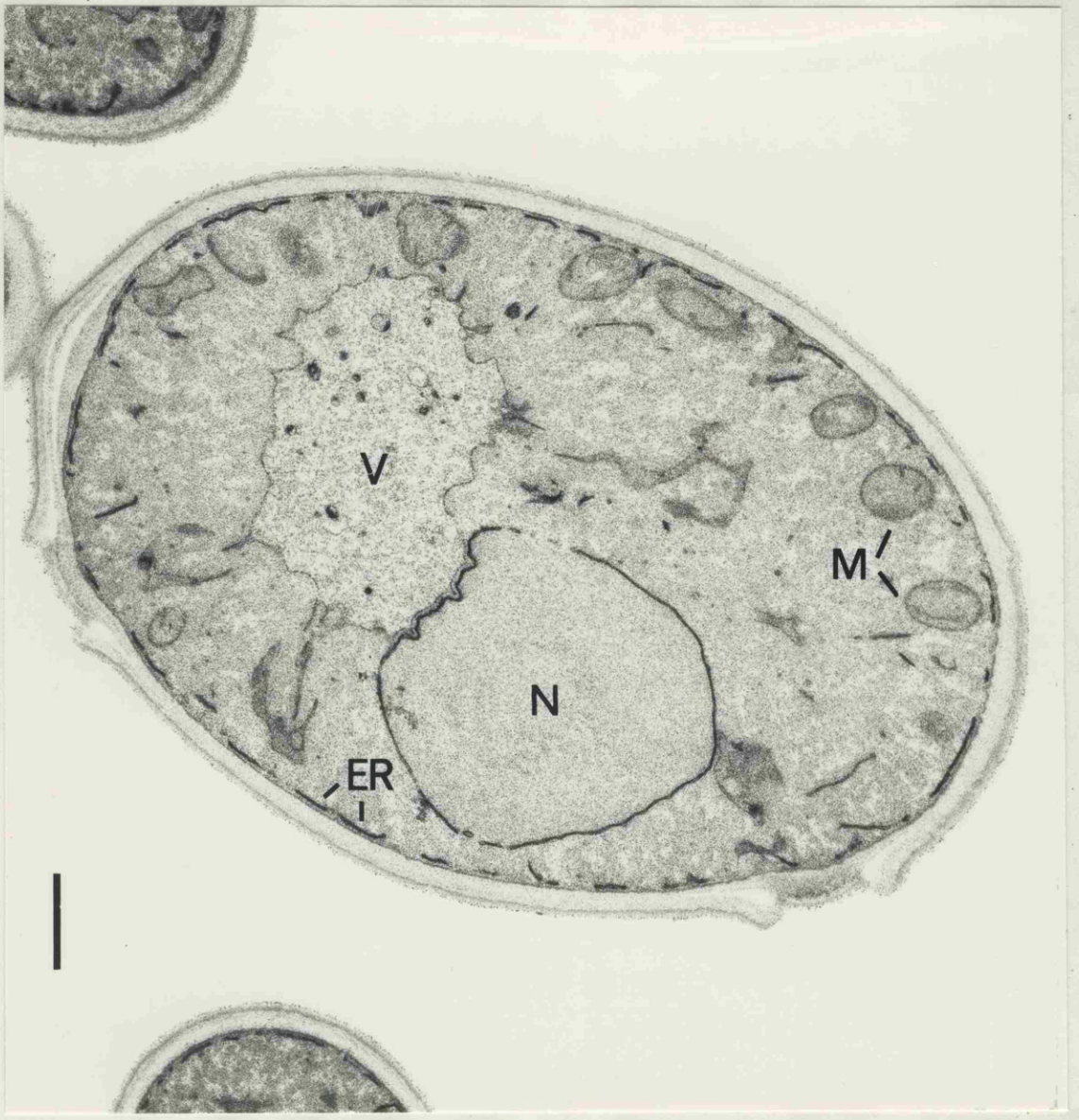


Plate 7. Developing ascus after 6h incubation in sporulation medium showing few structural changes from the vegetative phase except for an increase in the number of mitochondria (M) and the alignment of endoplasmic reticulum around the periphery of the cell. The bar represents 1μm.

Plate 8. Developing ascus after 12h in sporulation medium. The vacuole (V) is highly lobed as seen in tangential section, and a few electron-transparent vesicles (TV), bounded by a dense layer are seen in the cytoplasm. The bar represents 1µm.

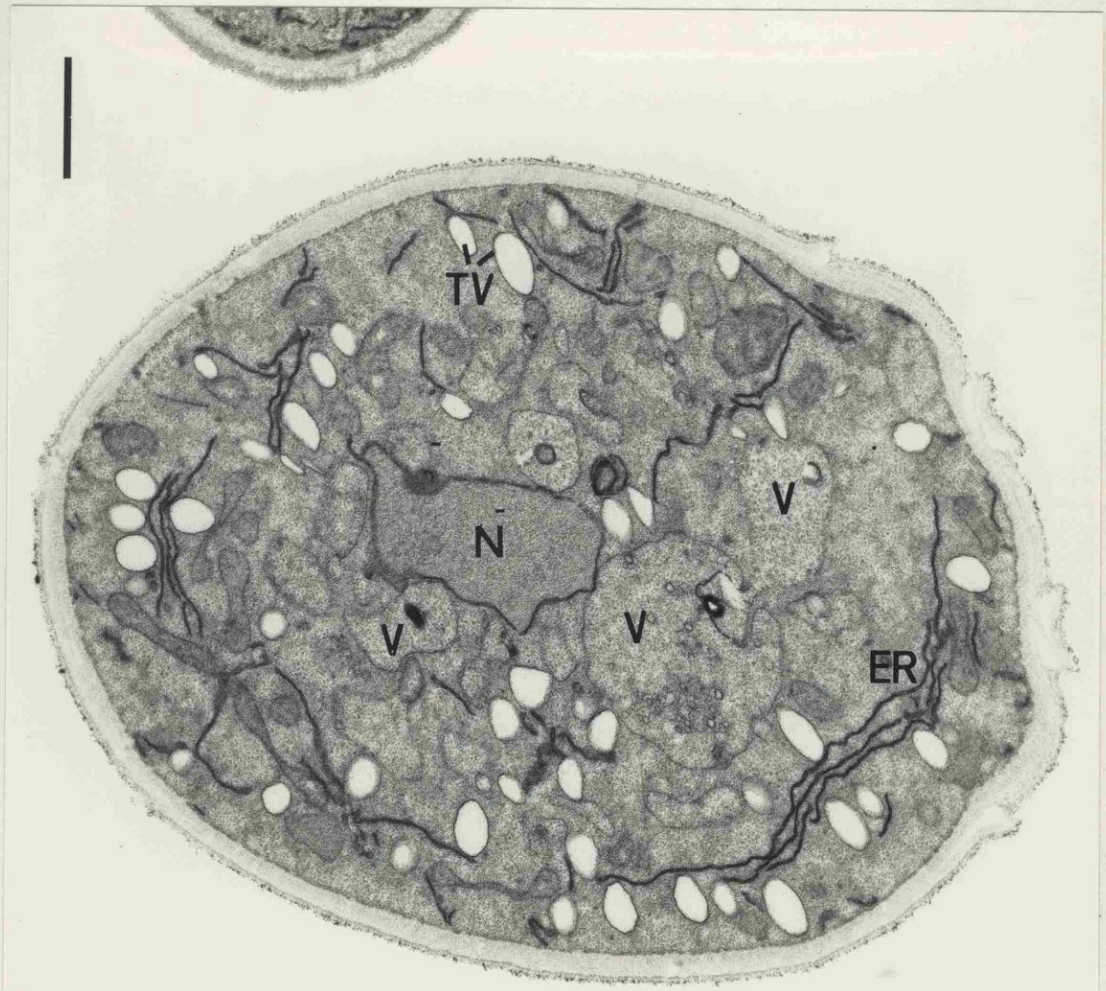


Plate 9 An ascus after 18h incubation in sporulation medium showing dramatic changes in cellular fine structure. Numerous electron-transparent vacuoles (TV) can be seen throughout the cell, an extensive membrane system is present (ER) and the vacuole has now fragmented resulting in several scattered vacuoles (V) some of which contain small membrane-bound vesicles.
The bar represents 1μm.

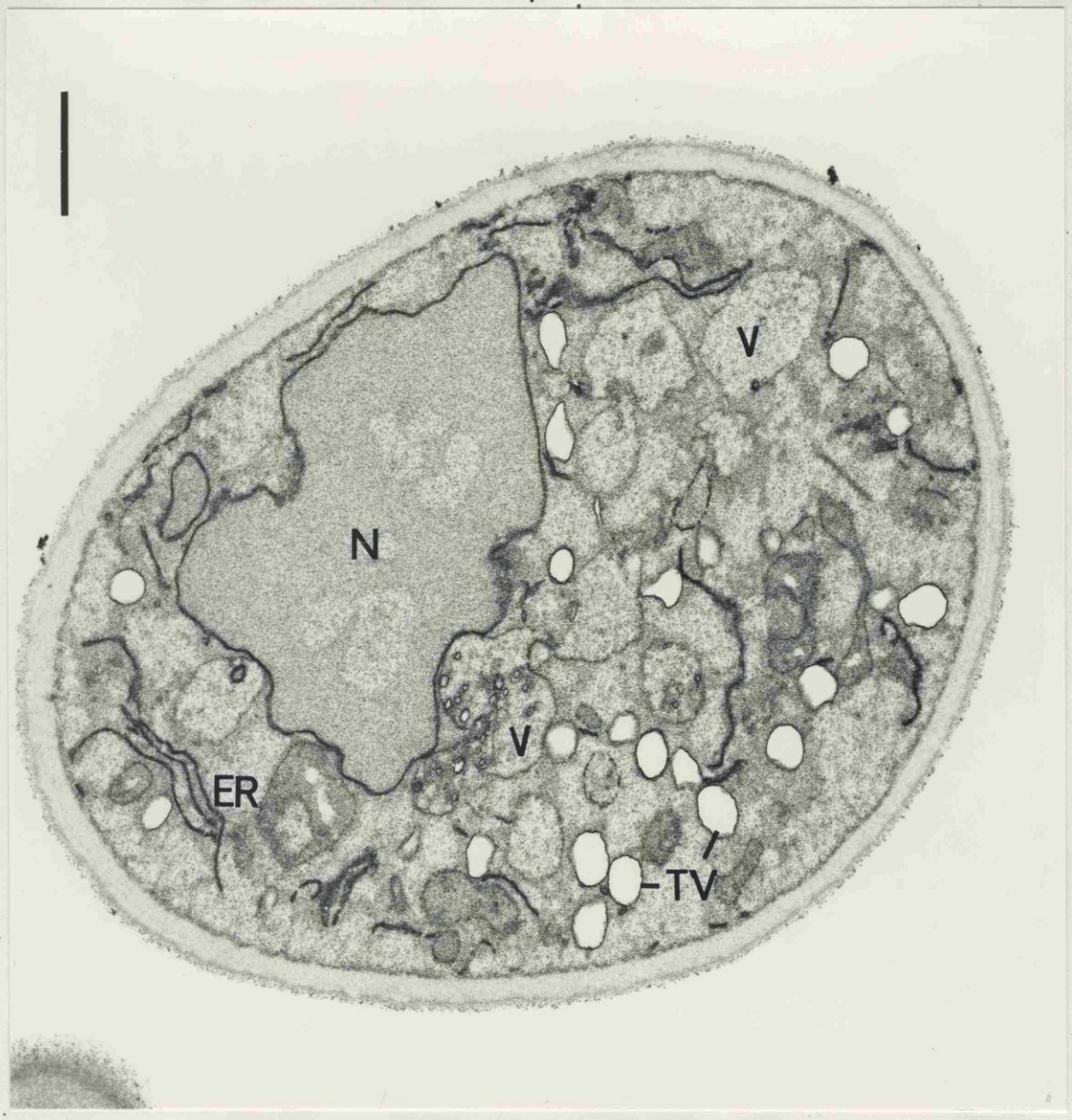


Plate 10. An ascus after 24h incubation in sporulation medium. Numerous electron-transparent vesicles (TV), vacuoles (V) and endoplasmic reticulum profiles (ER) are present throughout the cell. The outline of the nucleus is much less regular than previously. The bar represents 1µm.

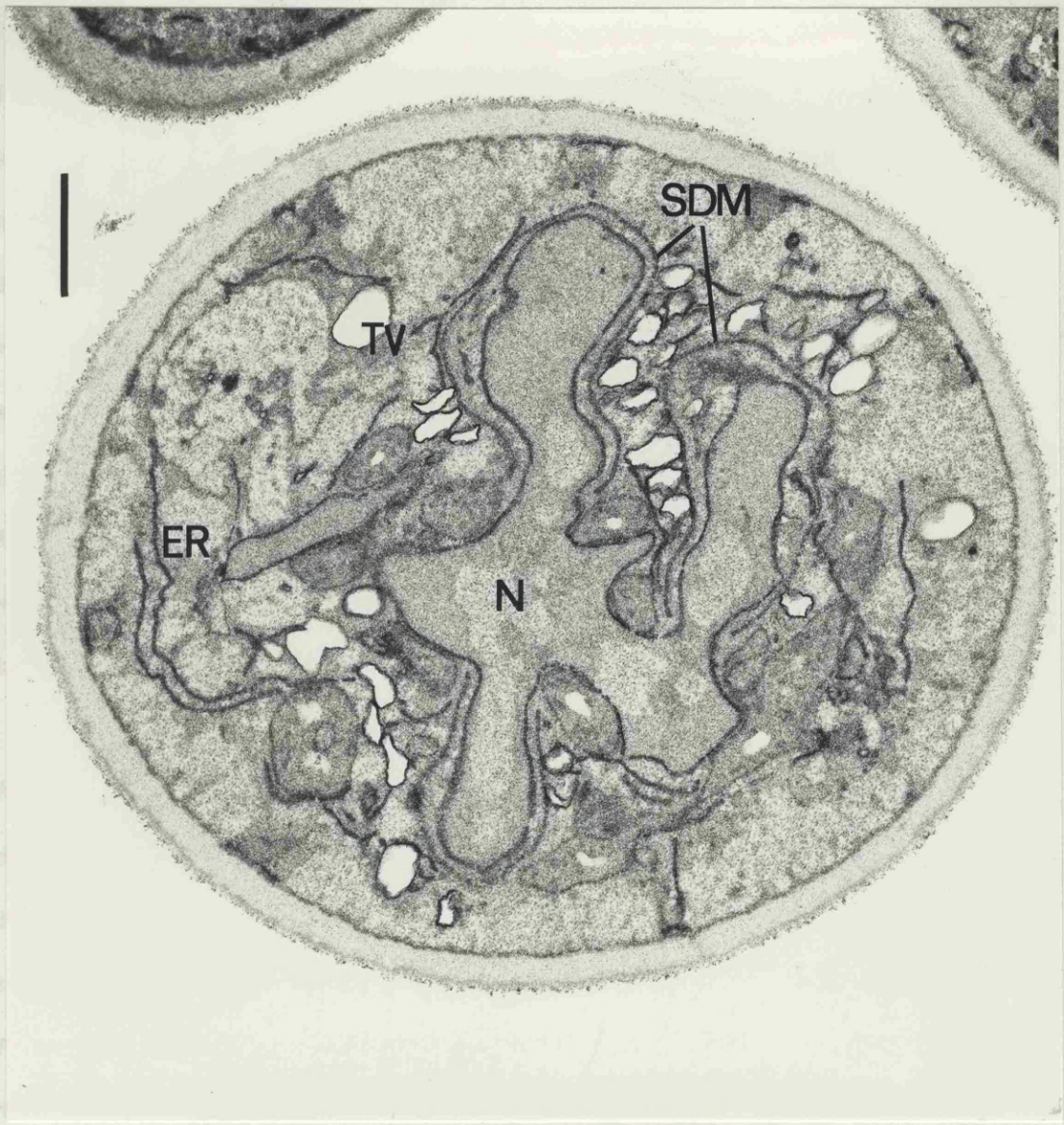


Plate 11. An ascus after 24h incubation in sporulation medium. The highly lobed nucleus (N) is apparently undergoing division, and the spore-delimitation membranes (SDM) can be seen enveloping the lobes of the nucleus in what are assumed to be the polar regions of the intranuclear spindle. The spindle microtubules are not preserved by potassium permanganate. Electron-transparent vesicles (TV) are aligned around and between the lobes of the nucleus. The bar represents 1µm.

picture of one of the nuclear lobes (Plate 12) demonstrates the double membrane nature of the spore delimiting membranes more clearly.

Irregular-shaped lipid vesicles are clustered on the outside of the spore-delimiting membranes and are especially numerous at points where adjacent lobes are close together (Plates 11, 12, 13). The spore-delimiting membranes extend further around the nuclear lobes until eventually the spore nucleus is cut off from the parent nucleus. The spore-delimiting membranes around the spore at the upper right in Plate 13 have nearly completed this process; the two spores in Plate 14 have completed this process. Cytoplasm, endoplasmic reticulum membranes and lipid vesicles are enclosed within the developing spore by its delimiting membranes.

A section through an immature spore (Plate 15) reveals that the spore-delimiting membranes have failed to envelop the spore completely leaving a small pore, at which point the two unit membranes which comprise the spore-delimiting membranes can be seen to be continuous. A certain amount of spore-wall material has already been deposited in the space between these two membranes, a process which continues as the spore wall matures. Before the spore wall develops these two membranes appear to be equally dense but, as the spores mature, the outer spore-investing membrane becomes more heavily stained, whereas the inner membrane, the spore plasma membrane, is less stained (compare Plates 12, 15, 16).

Several electron-transparent blebs are visible on the spore-investing membrane of many spores (Plate 15). Plate 16 shows bleb in detail. These blebs appear to be situated in the electron-transparent middle layer of the unit membrane which had swelled to many times its

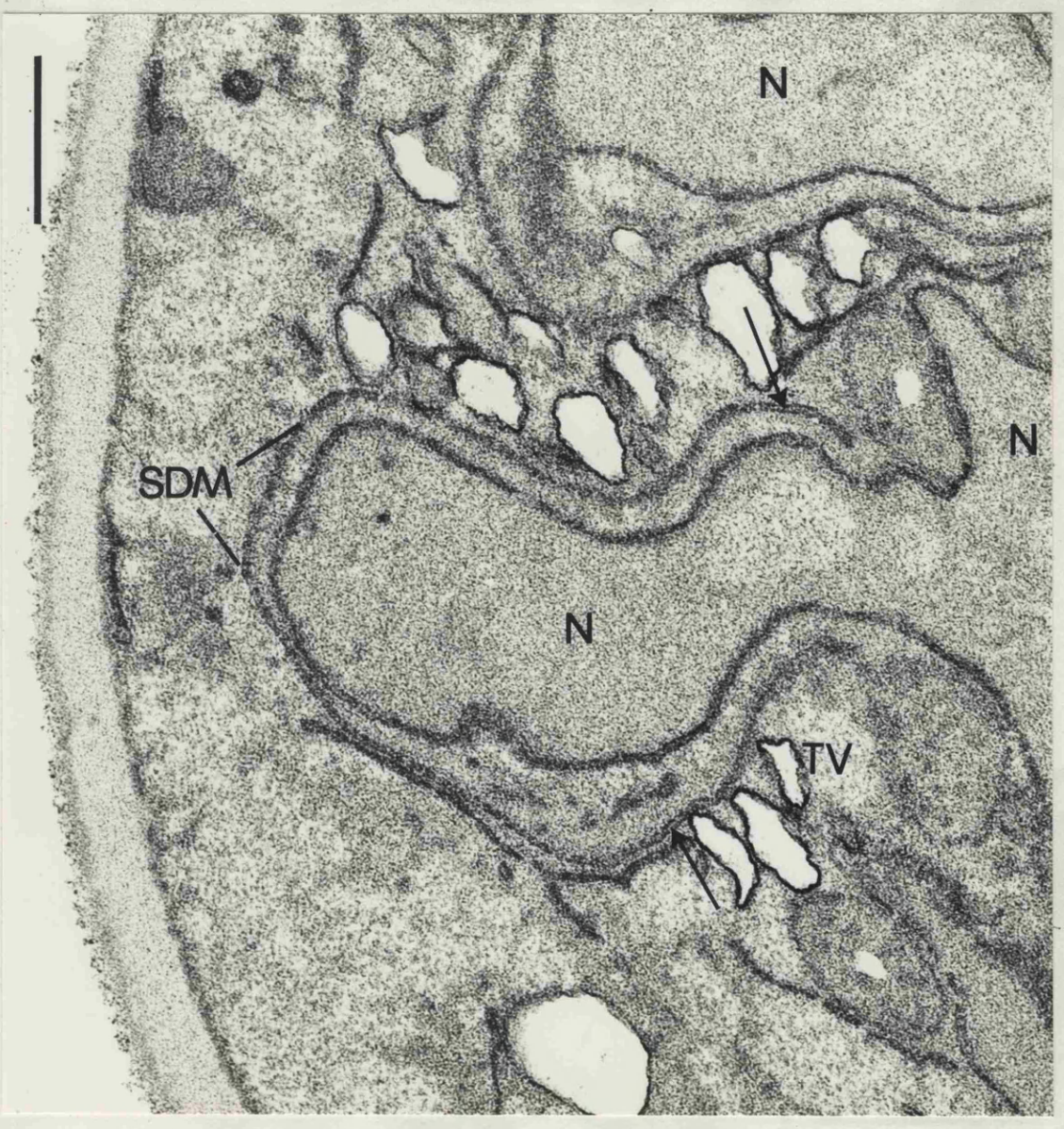


Plate 12. Part of an ascus after 24h incubation in sporulation medium. Spore-delimiting membranes (SDM) have begun to envelop the lobe of a multilobed, dividing nucleus (N). Transparent vesicles (TV) are associated with the spore-delimiting membranes which can be seen to be double (arrows). The bar represents 0.5µm.

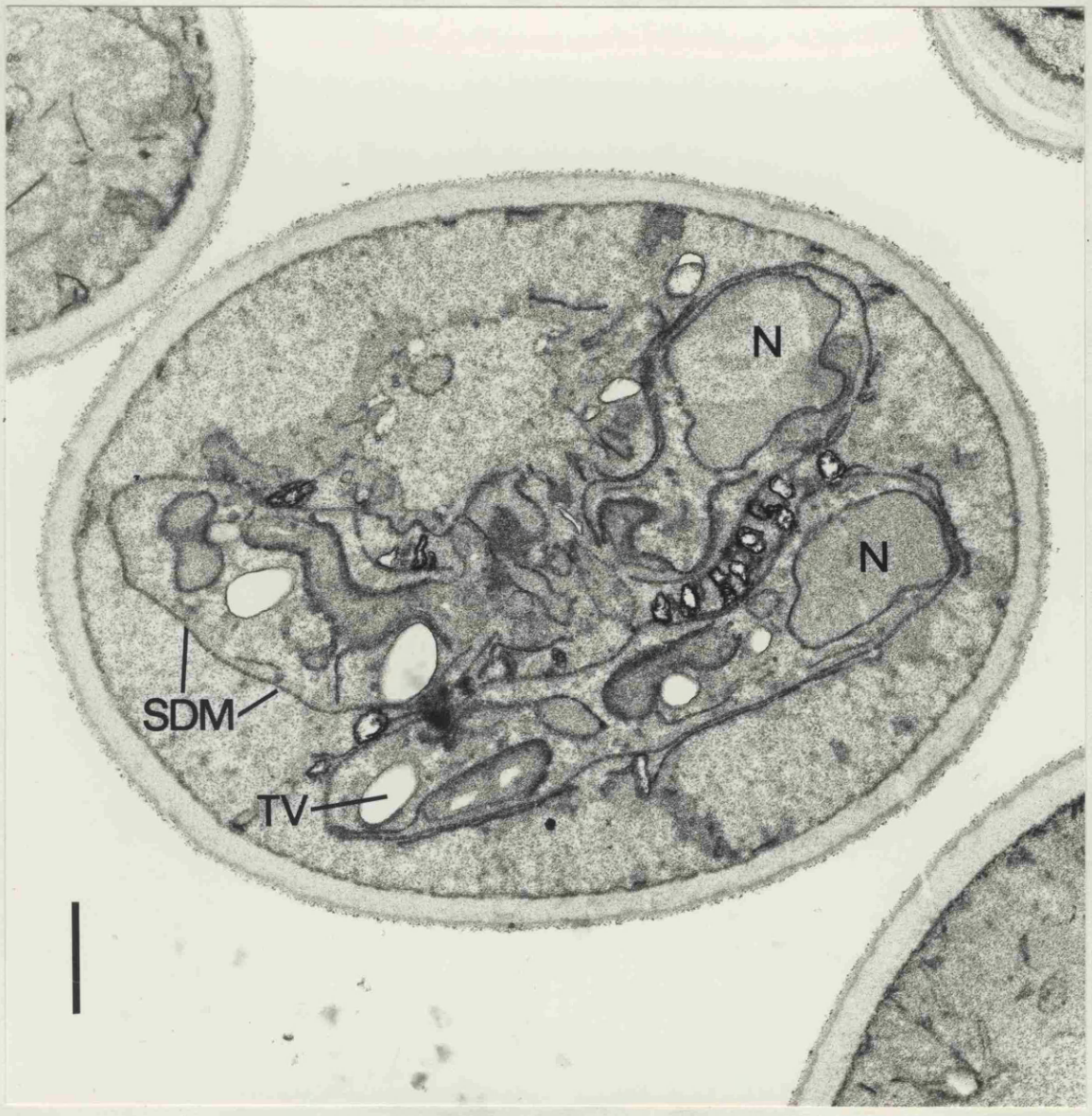


Plate 13. Another ascus after 24h incubation in sporulation medium. Delimitation of ascospores is nearly complete. Electron-transparent vesicles (TV) are enclosed within the spore-delimiting membranes (SDM). Smaller vesicles with electron-transparent centres and thick dense bounding layers are closely aligned between the developing spores. The bar represents 1μm.

Plate 14. Ascus containing two spores after 24h incubation in sporulation medium Delimitation of ascospores(AS) has just reached completion. Electron-transparent vesicles (TV) are present both within the cytoplasm of the ascus and of the spores. The bar represents 1µm.

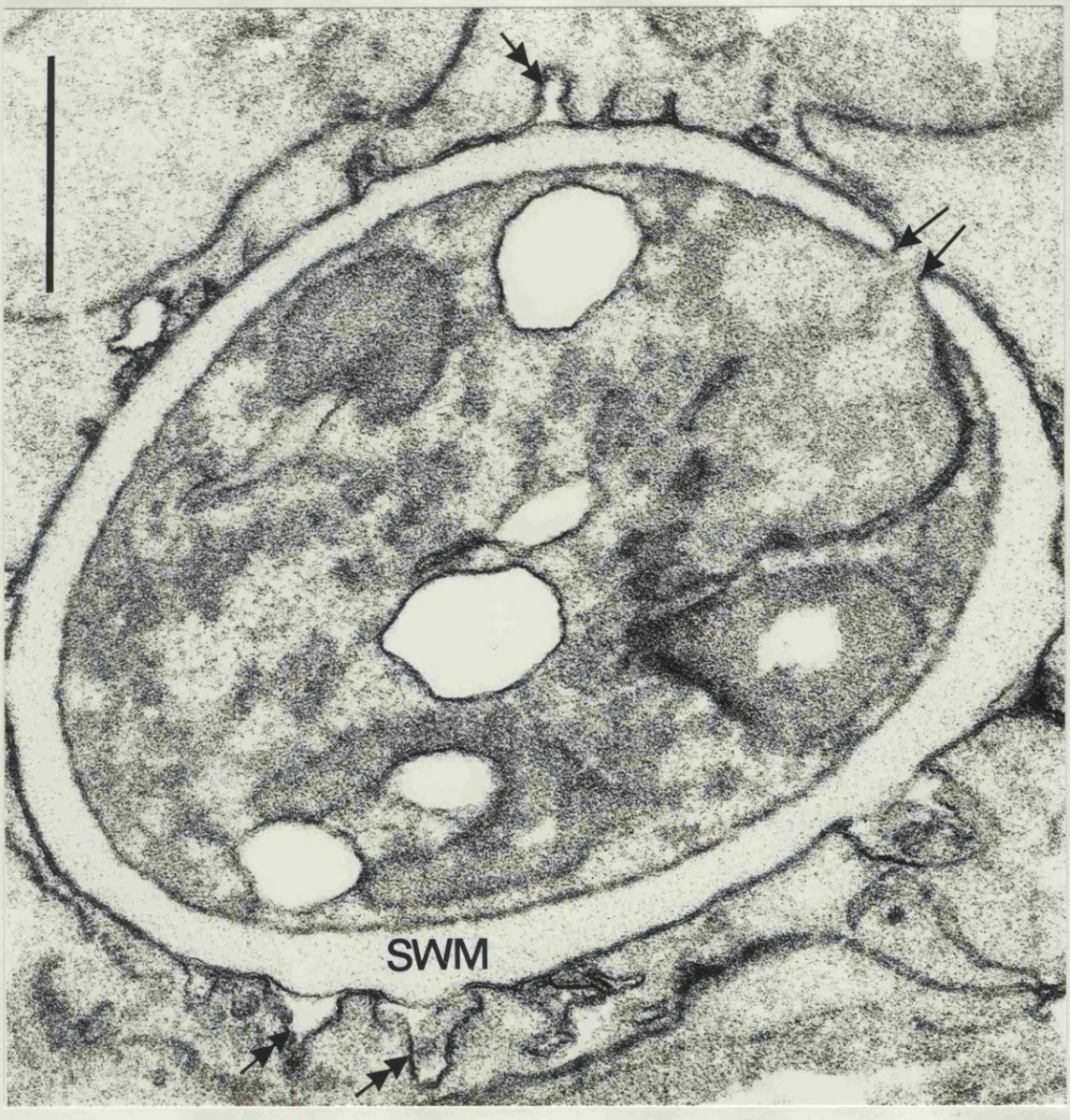


Plate 15. An incompletely delimited ascospore after 24h incubation in sporulation medium. Deposition of spore-wall material (SWM) has already occurred between the two delimiting membranes which are seen to be continuous (arrows). The close association of transparent (lipid) vesicles with the outer of the two delimiting membranes can be seen (double arrows). The bar represents 0.5µm.

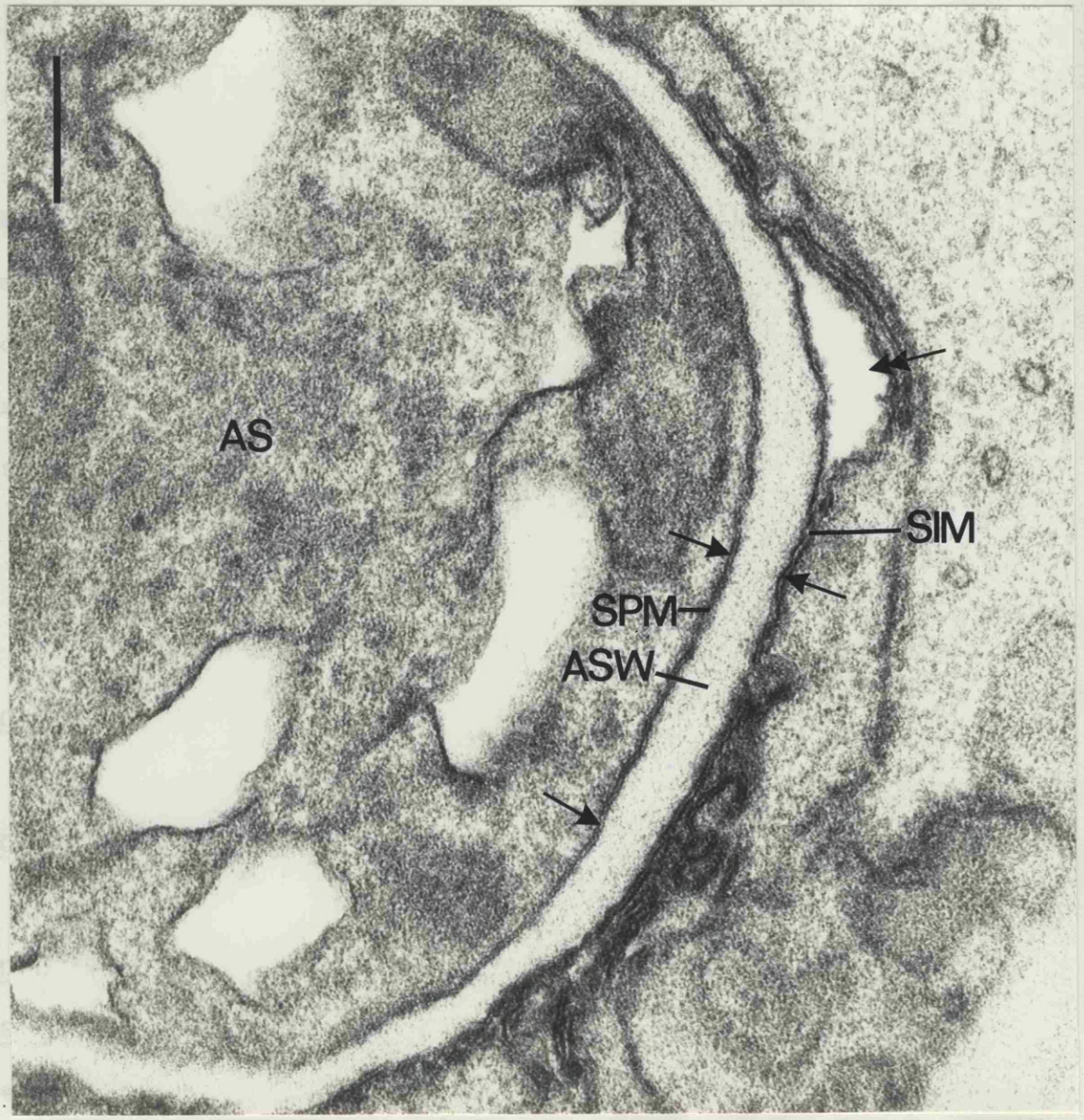


Plate 16. Part of a maturing ascospore (AS) after 24h incubation in sporulation medium. The unit-membrane nature of both the inner spore plasma membrane and the outer spore-investing membrane (SIM) is clearly seen (arrows). Material of the ascospore wall (ASW) has been deposited between these two membranes and a lipid vesicle can be seen closely associated with the spore investing membrane (double arrows). The bar represents 0.25μm.

original thickness. This central layer is recognised as being the location of the membrane phospholipids, and it would seem likely that these blebs are composed mainly of lipid. Possibly the blebs are formed from the lipid vesicles seen in Plates 11 and 12.

Plates 17 and 18 show immature asci at T_{24}. The surfaces of ascospores at T_{24} are covered with membraneous debris, possibly the remains of blebs which have emptied and collapsed. The epiplasm of these two asci is also full of membranes profiles, membrane-bound vesicles and the remains of the vacuole.

CHANGES IN LIPID COMPOSITION AND SYNTHESIS DURING ASCUS DEVELOPMENT

Changes in composition

Dry weight of developing asci. There is no vegetative growth of Sacch. cerevisiae in sporulation medium although cells increase in dry weight (Croes, 1967a; Esposito et al., 1969) and volume (Croes, 1967a) as ascospore formation proceeds. Cells and asci were dried by two methods, namely freeze-drying and drying under reduced pressure at 80°C (Fig. 1). Freeze-dried asci increased in weight, as development proceeded, by about 150% whereas heat-dried asci increased in weight by only 70%. Clearly freeze drying became progressively less effective as a means of removing water from asci during the course of sporulation. This inability to remove water from developing asci by freeze drying did not increase linearly during sporulation but occurred during two distinct periods, the first one from T_0 to T_{12} and the second from T_{30} to T_{48} (Fig. 2).

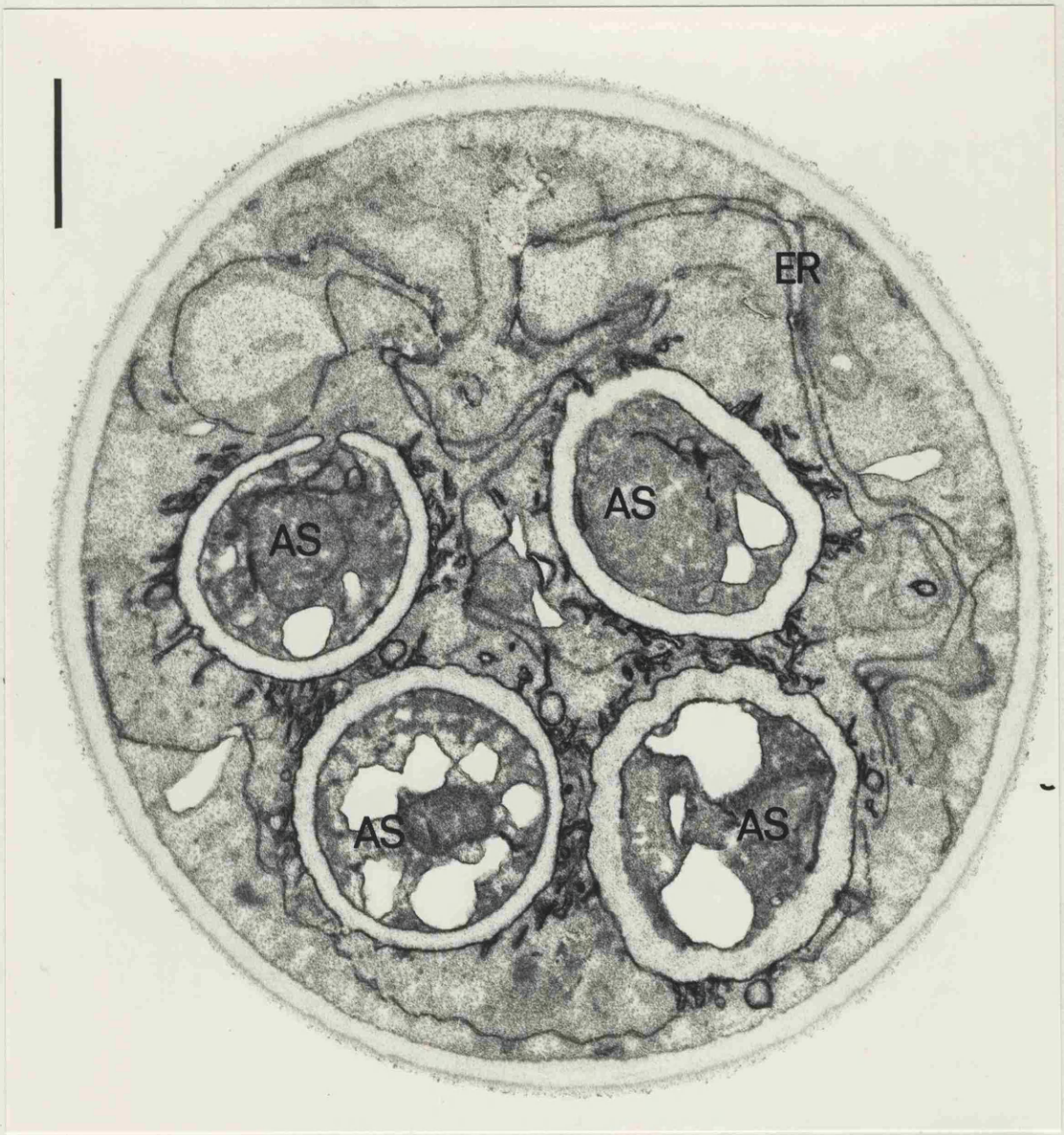


Plate 17. An ascus containing four maturing ascospores (AS) after 24h incubation in sporulation medium. Around the outside of each ascospore is a mass of membraneous debris, probably derived from the collapsed lipid vesicles which have been incorporated in the developing spore wall. Membrane profiles of endoplasmic reticulum (ER) are still prominent in the epiplasm. The bar represents 1µm.

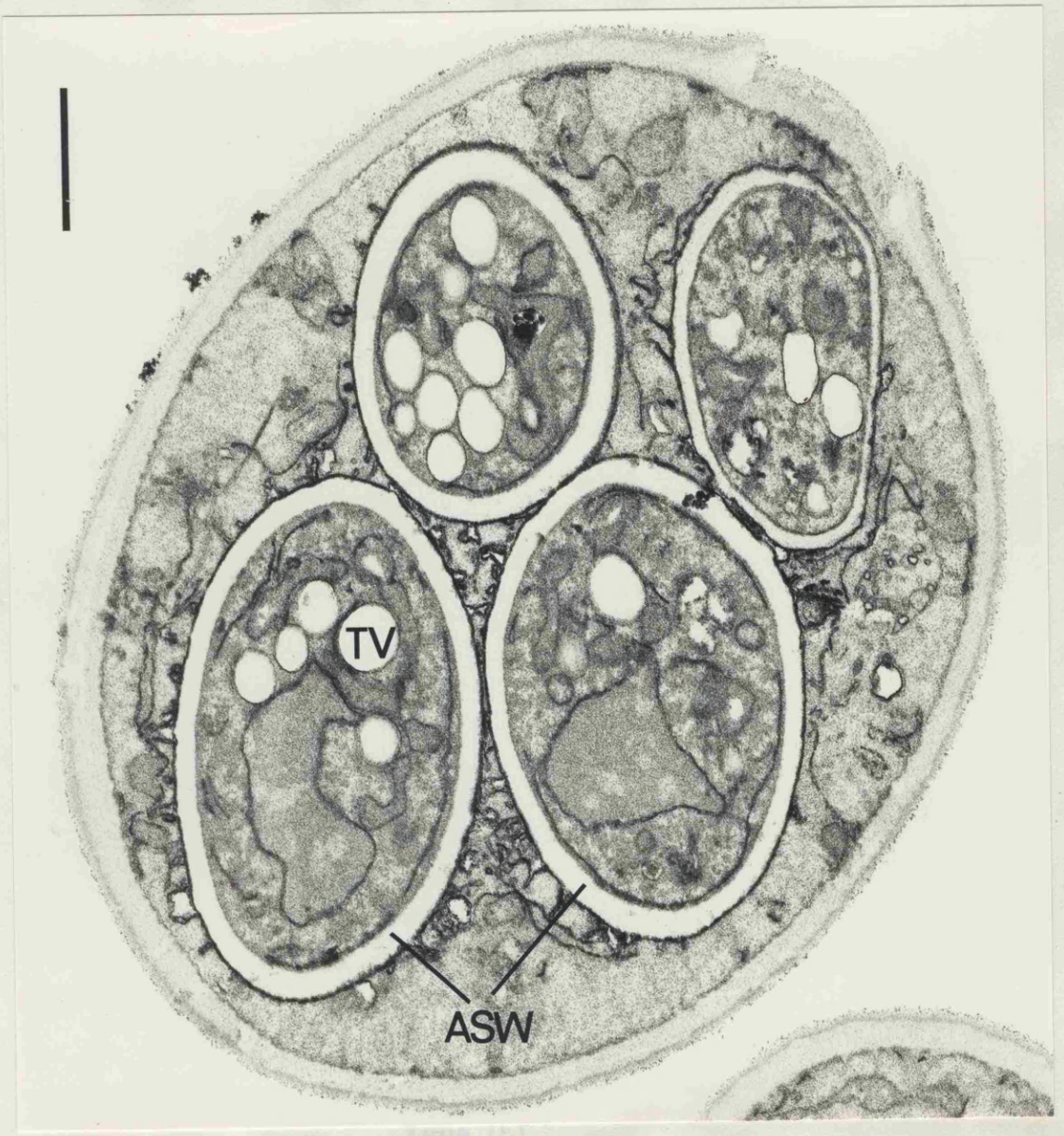


Plate 18. Another ascus containing four maturing ascospores after 24h incubation in sporulation medium. Around each ascospore a well defined wall (ASW) has developed and each spore contains at least one transparent vesicle (TV). The bar represents 1µm.

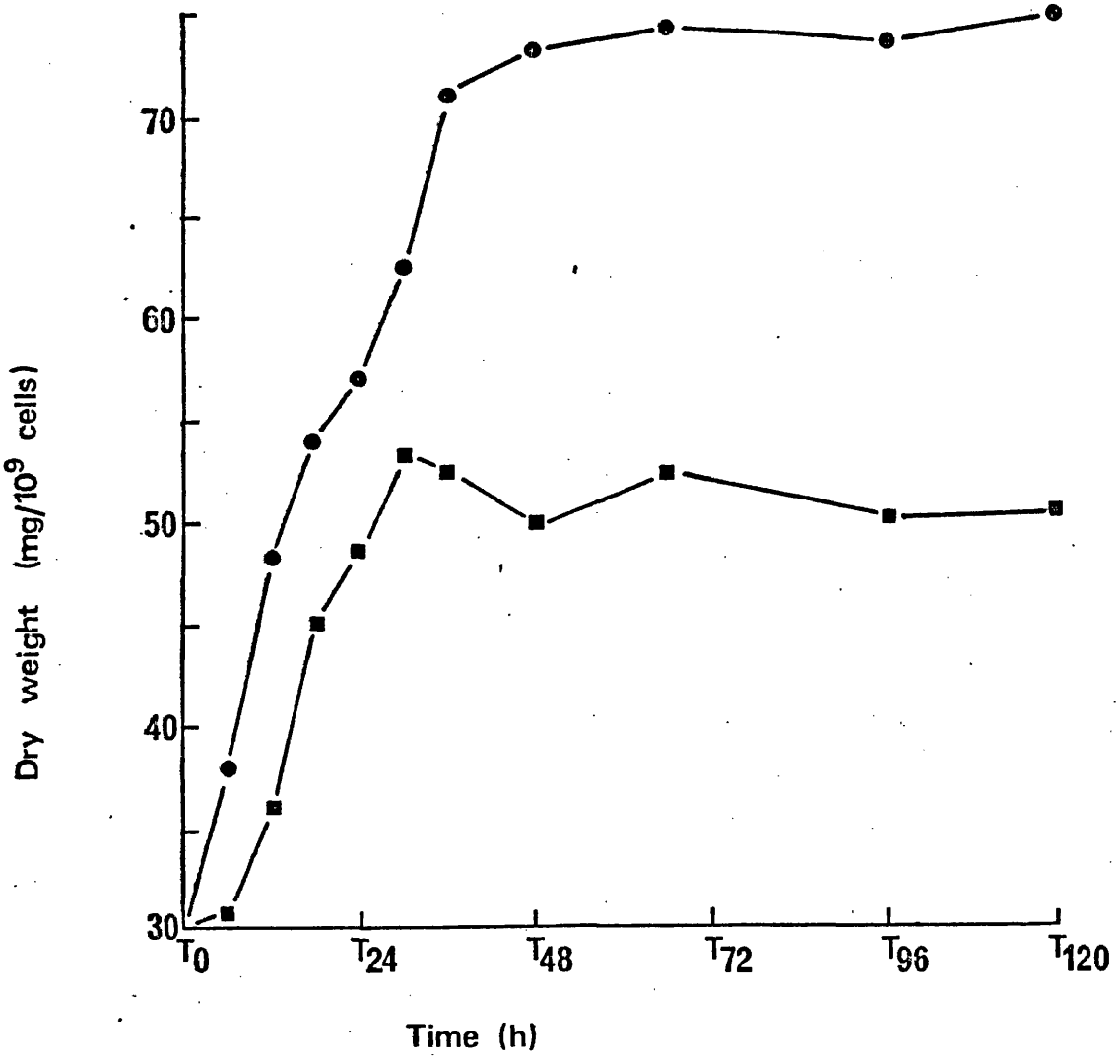


Figure 1. Changes in dry weight of Saccharomyces cerevisiae during ascus formation.

●—● cells dried by freeze drying.

■—■ cells dried at 80°C under reduced pressure (see text).

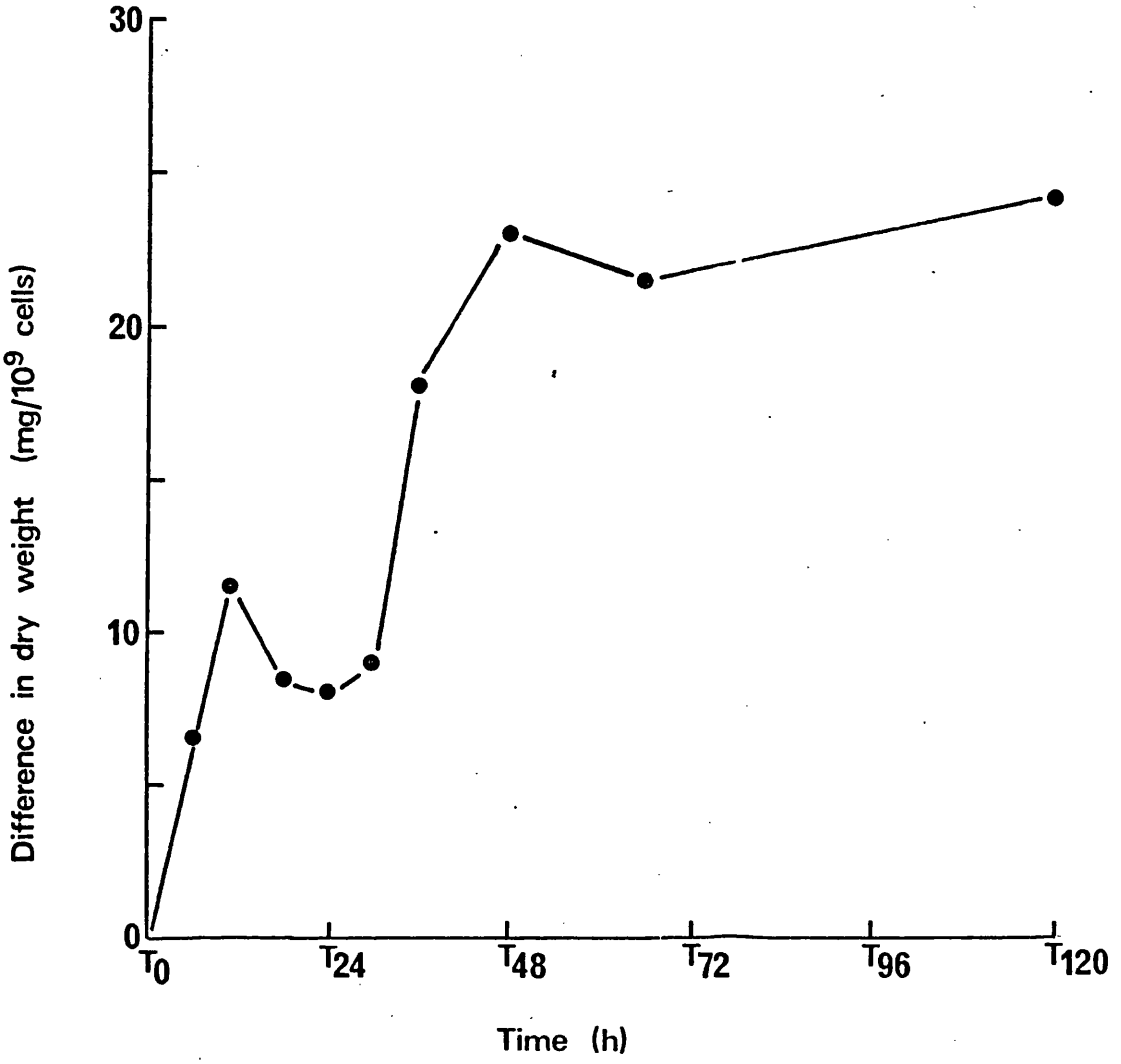


Figure 2. Changes in water-retaining capacity of cells of Saccharomyces cerevisiae during ascus formation. Water-retaining capacity was assessed as the difference in weight between heat-dried cells and freeze-dried cells.

Extraction of lipids from cells and developing asci. Lipids were extracted from cells and developing asci with hot ethanol followed by three extractions with chloroform-methanol (1:1 v/v; see Methods section). This method gave the most reproducible results. Other methods were tried, including that of Longley et al. (1968) and a method which involved disrupting the cells with glass beads prior to extraction with solvents. These methods proved to be much less satisfactory.

Enzymic degradation of lipids is likely to occur to some extent during any process of lipid extraction. Lipids extracted by ethanol followed by chloroform-methanol have a low content of diacylglycerols, free fatty acids and lysophospholipids, compounds which would be amongst the products of lipid degradation. The other methods produced lipid extracts which had a higher content of these breakdown products. The contents of lysophospholipids were especially high.

Changes in lipid composition during ascus development. Several phospholipids and non-polar lipids were detected on thin-layer chromatograms of extracts from cells, developing asci and mature asci. The non-polar lipids included monoacylglycerols, diacylglycerols triacylglycerols, sterols, sterol esters, squalene and hydrocarbons. Chromatographic separation of phospholipids was performed by two dimensional thin-layer chromatography. Six phospholipids were identified which included large proportions of phosphatidylcholine, phosphatidyl-ethanolamine, phosphatidylinositol and smaller proportions of phosphatidylserine, phosphatidic acid and cardiolipin. Examination of extracts of cells before, during and after sporogenesis revealed that there were no detectable qualitative changes in the phospholipid

composition as a result of sporulation.

Assays of total lipid and individual lipid fractions from vegetative cells and developing asci are shown in Figs. 3, 4, 5, 6, 7, 8, 9 and 10. Monoacylglycerols were detected by thin-layer chromatography but there was an insufficient amount of this class of lipid present to assay accurately. Hydrocarbon was not assayed and it was subsequently shown that the small amounts present in extracts (about 5mg/extract) were contaminants of the ethanol used in the lipid-extraction procedure.

As noted earlier there is an increase in dry weight of the developing asci but no increase in cell number during sporulation. Consequently it was decided to express the results of all analyses in terms of cell number, rather than dry weight. A population of 10^9 cells was found to be a convenient size for quoting analytical data.

The weight of total lipid rapidly increases to a maximum during the period T_0 to T_{24} and then falls steadily toward T_{120} (Fig. 3) This increase in total lipid is closely followed by an increase in the contents of phospholipids, sterol esters and triacylglycerols (Figs. 4, 5, 6). The contents of sterol ester and triacylglycerol continued to increase in a linear fashion up to T_{120}. The content of phospholipid remained fairly constant between T_{24} and T_{120} while the contents of other lipid fractions, namely sterols, diacylglycerols, free fatty acids and squalene increased during sporogenesis (Figs. 7, 8, 9, 10).

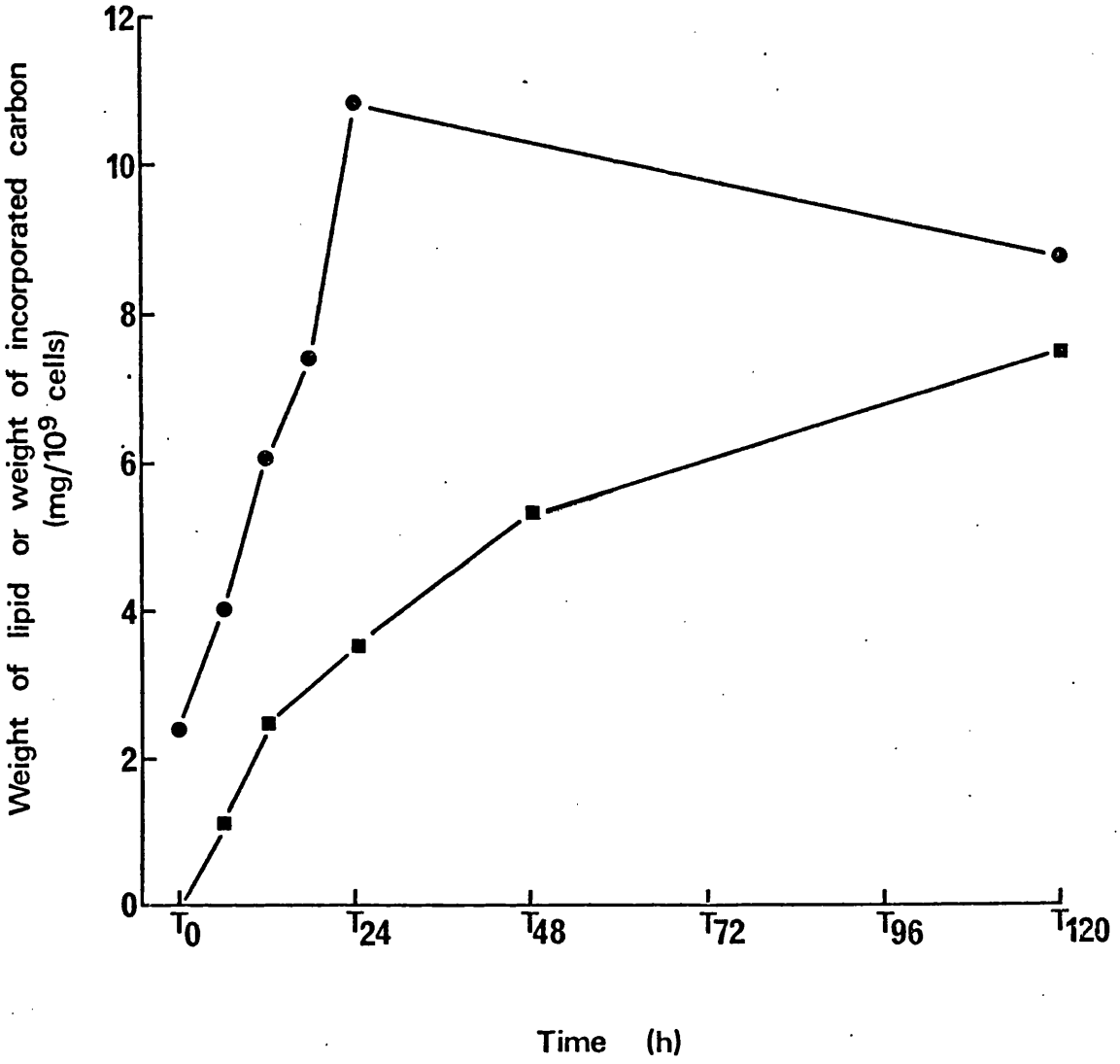


Figure 3. Changes in the weight of total lipid and in the extent of incorporation of acetate carbon into total lipid of cells of Saccharomyces cerevisiae during ascus formation.

●—● changes in the weight of total lipid.

■—■ incorporation of acetate carbon into total lipid when $(U\text{-}\ ^{14}C)$ acetate was included in the sporulation medium.

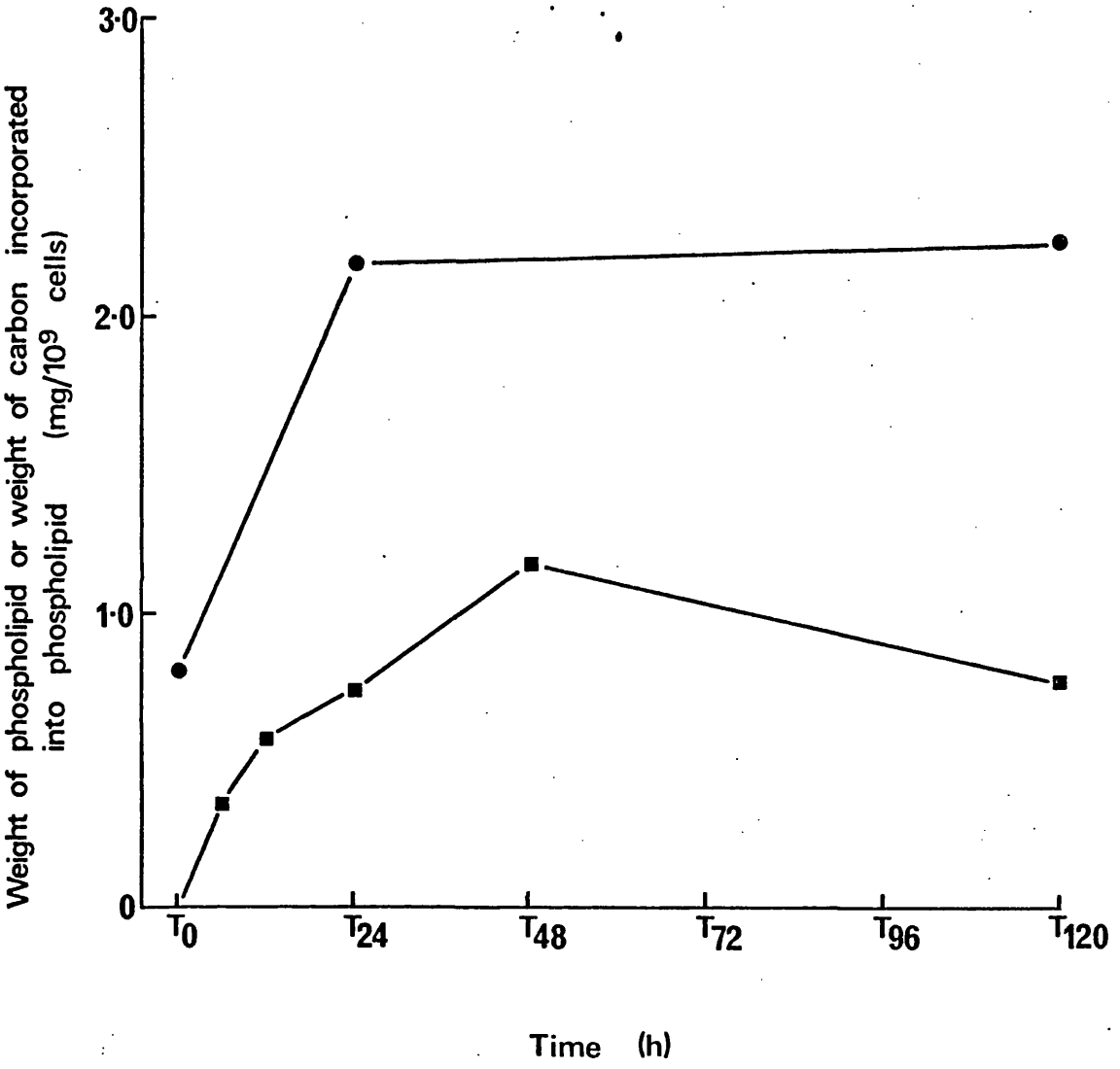


Figure 4. Changes in the content of phospholipids and the extent of incorporation of acetate carbon into phospholipids of cells of Saccharomyces cerevisiae during ascus formation.

●——● variations in phospholipid content.

■——■ incorporation of acetate carbon into phospholipids when $(U\text{-}\ ^{14}C)$ acetate was included in the sporulation medium.

The SEM value for each point was less than 10%.

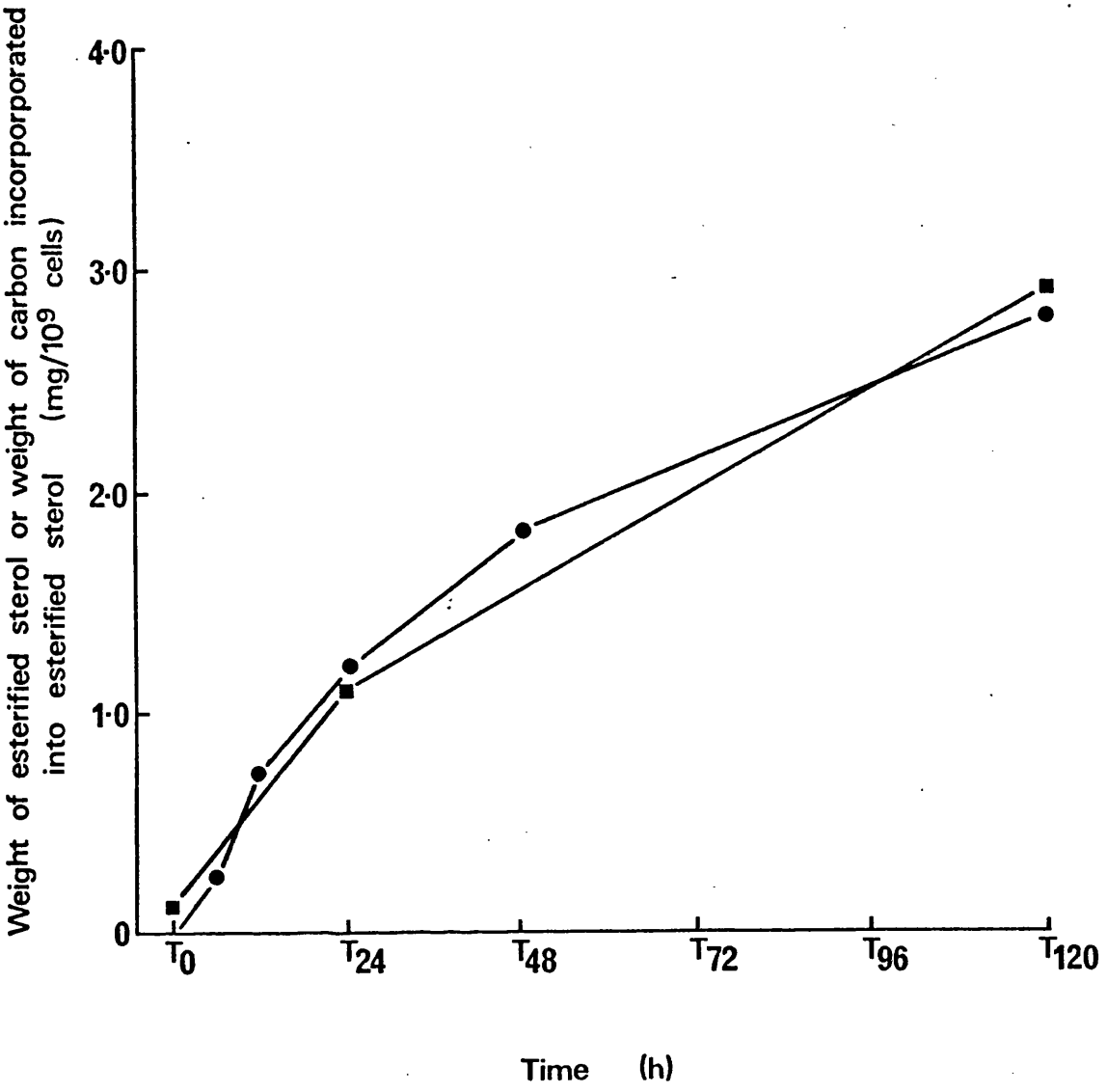


Figure 5. Changes in the content of esterified sterols and the extent of incorporation of acetate carbon into esterified sterols of cells of Saccharomyces cerevisiae during ascus formation.

■——■ variation in sterol ester content.

●——● incorporation of acetate carbon into sterol esters when $(U\text{-}\ ^{14}C)$ acetate was incorporated in the sporulation medium.

The SEM value of each point was less than 12%.

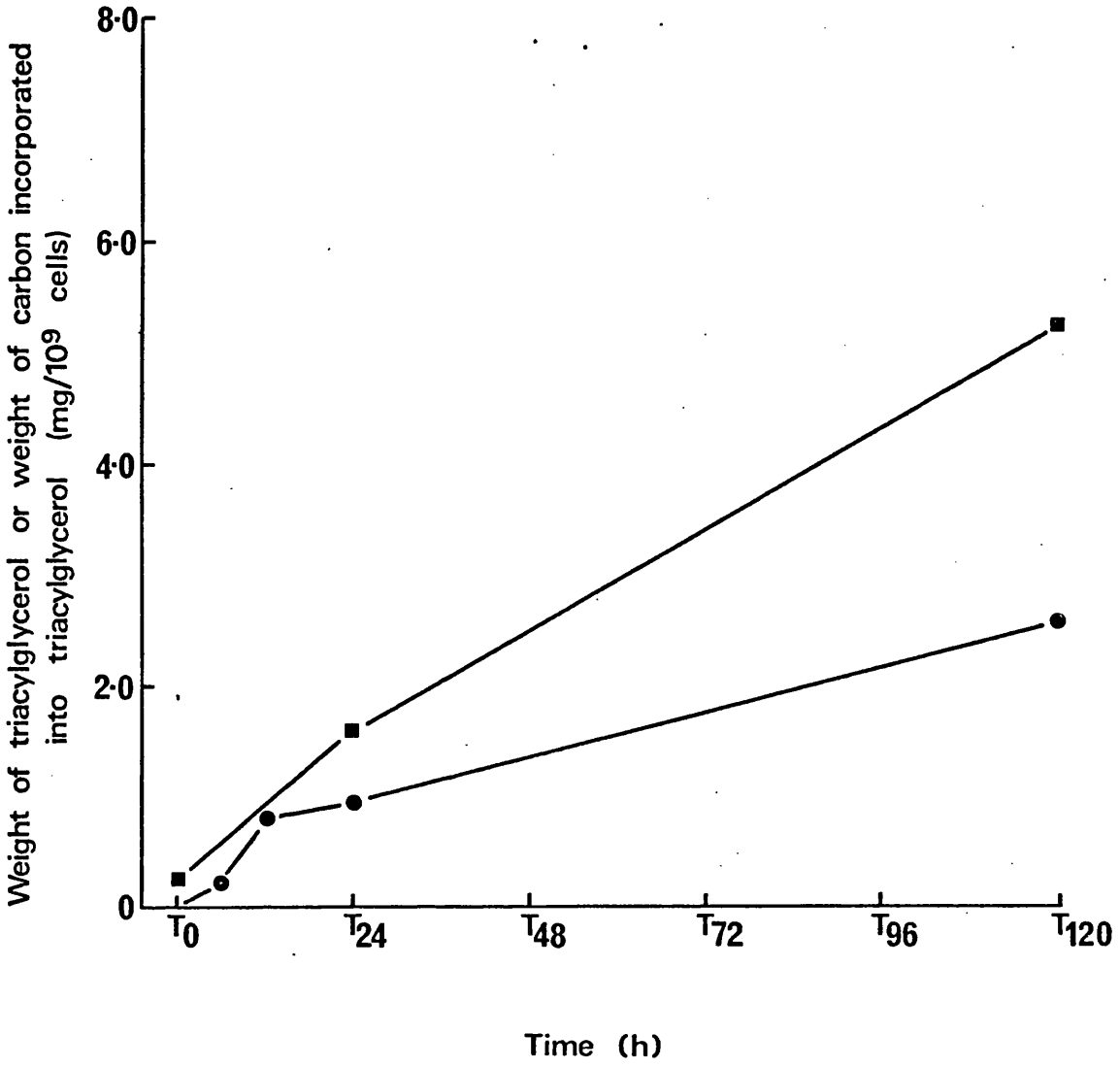


Figure 6. Changes in the content of triacylglycerols and the extent of incorporation of acetate carbon into triacylglycerols of cells of Saccharomyces cerevisiae during ascus formation.

■——■variation in triacylglycerol content.

●——●incorporation of acetate carbon into triacylglycerols when $(U-\ ^{14}C)$ acetate was included in the sporulation medium.

The SEM value of each point was less than 8%.

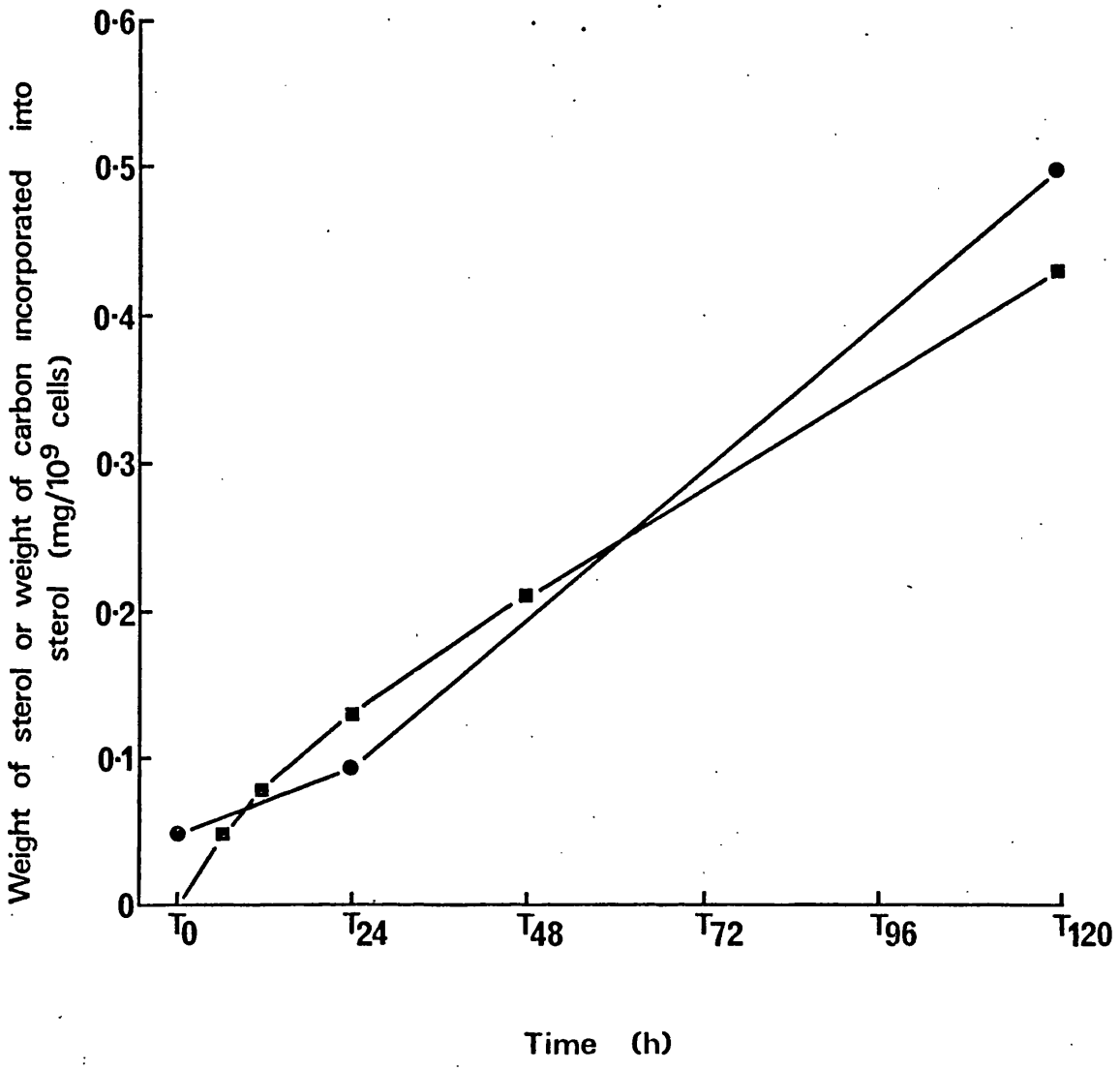


Figure 7. Changes in the content of sterols and the extent of incorporation of acetate carbon into sterols of cells of Saccharomyces cerevisiae during ascus formation.

●——● variation in sterol content.

■——■ incorporation of acetate carbon into sterol when $(U\text{-}{}^{14}C)$ acetate was included in the sporulation medium.

The SEM value of each point was less than 10%.

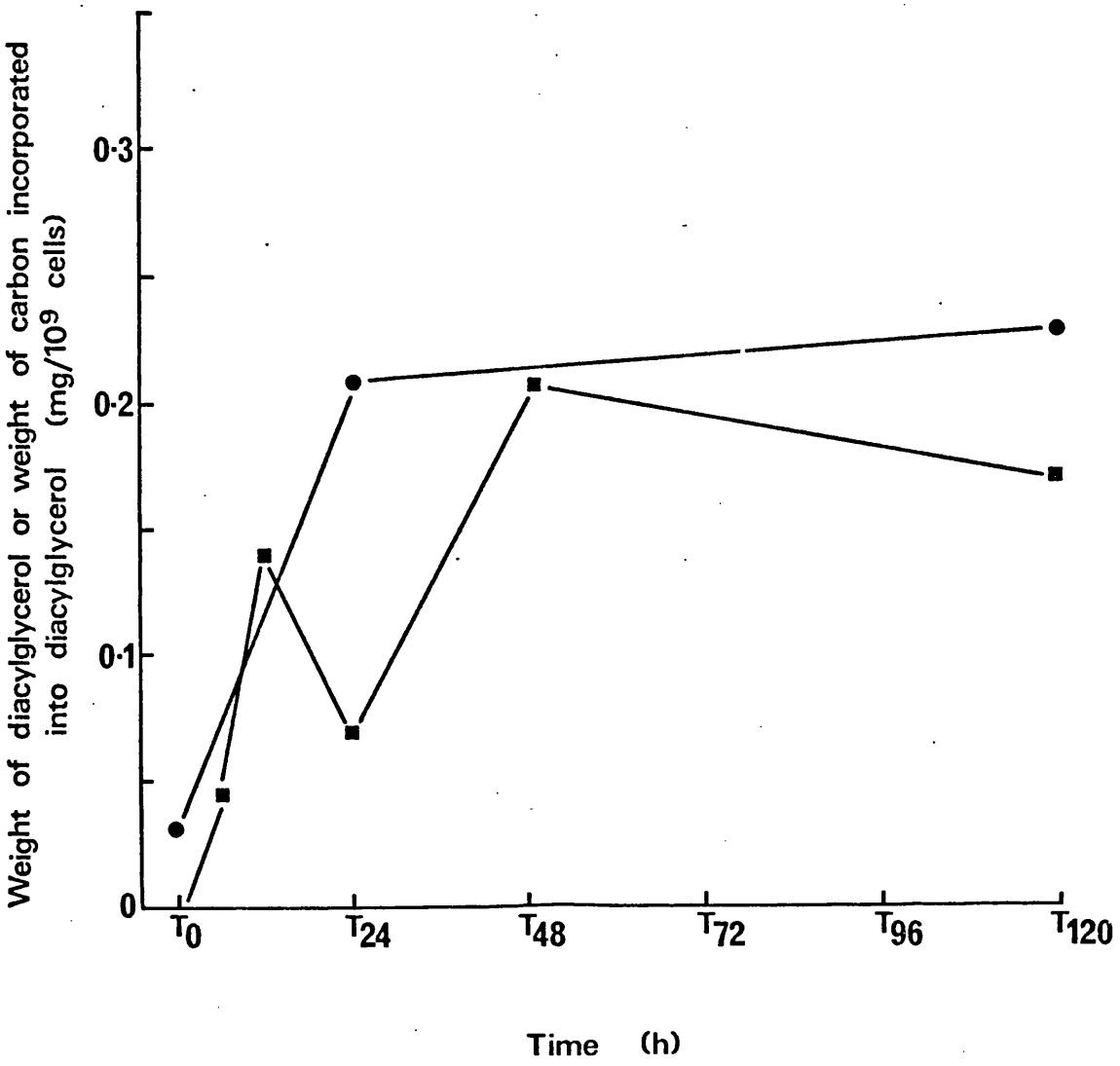


Figure 8. Changes in the content of diacylglycerols and the extent of incorporation of acetate carbon into diacylglycerols of cells of Saccharomyces cerevisiae during ascus formation.

●—● variation in diacylglycerol content.

■—■ incorporation of acetate carbon into diacylglycerol when $(U-{}^{14}C)$ acetate was included in the sporulation medium.

The SEM value of each point was less than 15%.

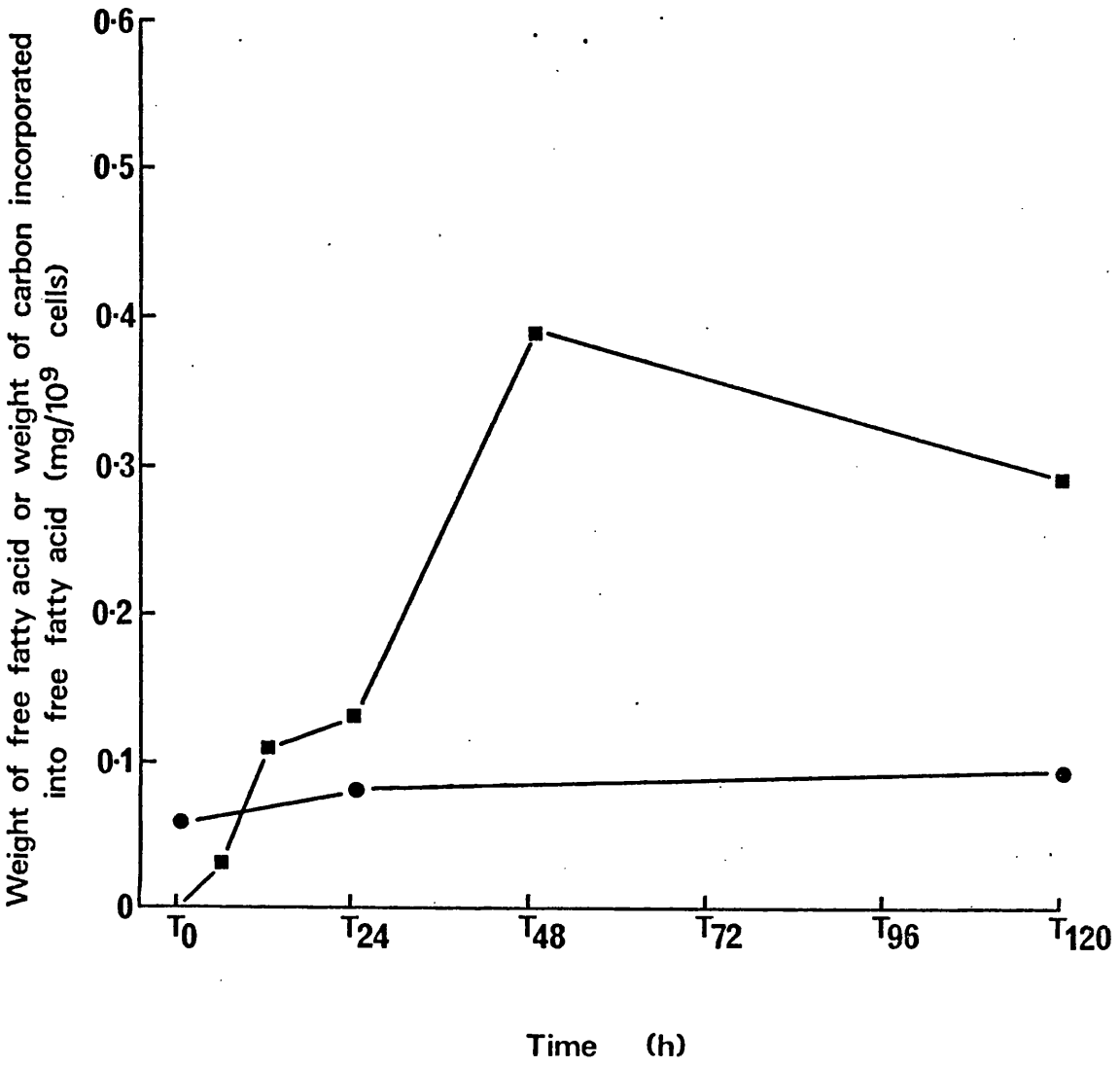


Figure 9. Changes in the content of free fatty acids and the extent of incorporation of acetate carbon into free fatty acids of cells of Saccharomyces cerevisiae during ascus formation.

●——● variation in free fatty acid content.

■——■ incorporation of acetate carbon into free fatty acids when $\left(U-{}^{14}C\right)$ acetate was included in the sporulation medium.

The SEM value of each point was less than 15%.

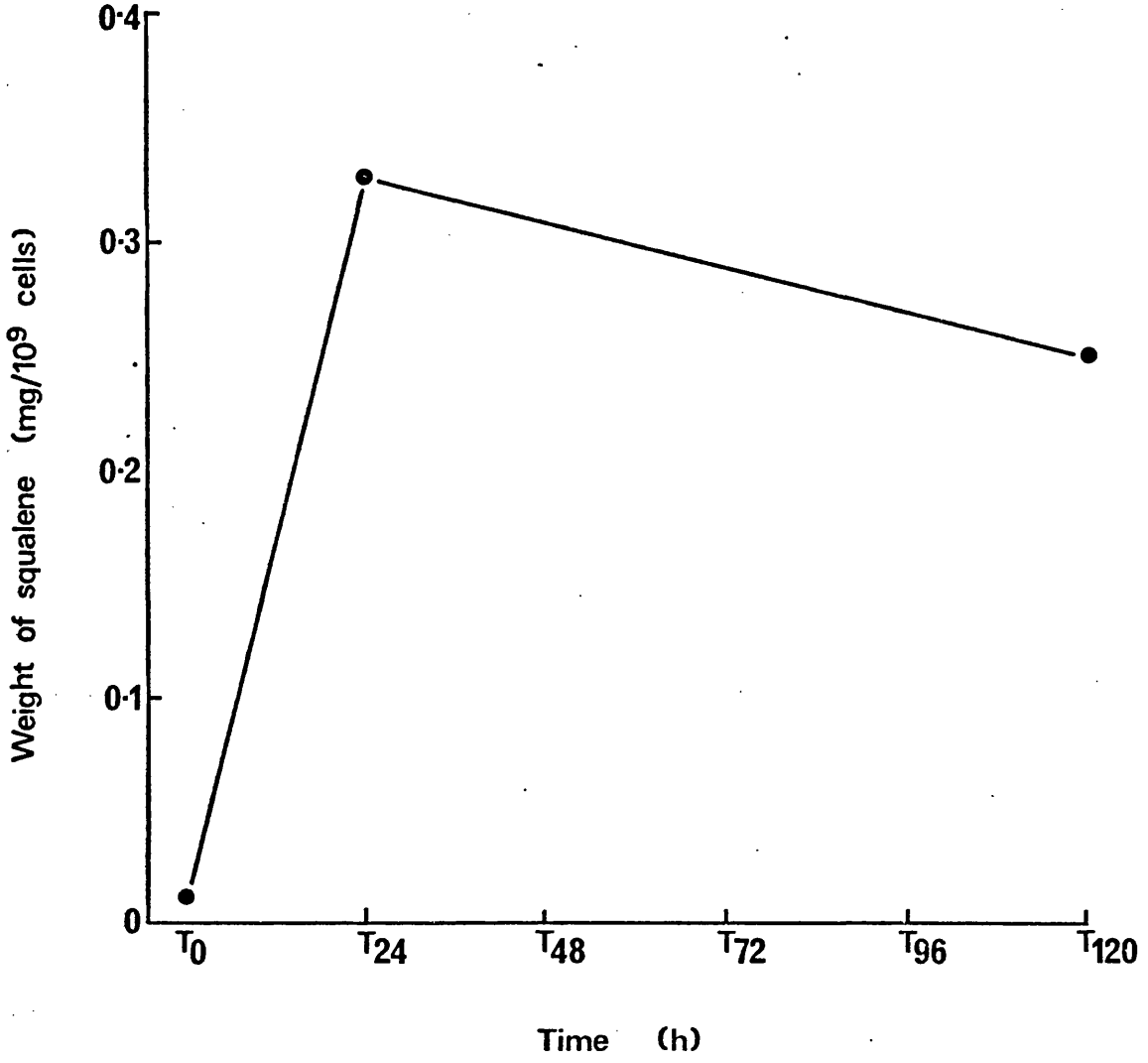


Figure 10. Variation in the squalene content of Saccharomyces cerevisiae during ascus formation.

The SEM value of each point was less than 10%.

Fatty-acid composition of lipids in developing asci. Gas-liquid chromatography of fatty acids obtained from lipid extracts indicated that the bulk of the fatty-acid residues are C_8 to C_{18} saturated and mono-unsaturated acids. All of the even-numbered fatty acids in this range were detected, but some of the odd-numbered acids, namely C_{11} and C_{17} were not detected. There was an increase in the percentage molar of unsaturated-acid residues during sporogenesis (Table 2). This is attributable to an increase in the relative amounts of $C_{16:1}$ and $C_{18:1}$ residues and a decrease in the amount of $C_{16:0}$ residues. The proportion of $C_{18:0}$ residues remained fairly constant (Table 2).

Several environmental factors including growth temperature, dissolved oxygen tension and limitation of substrate, have been shown to affect the fatty-acid composition of lipids in many organisms, including higher animals and plants (for a review see Farrell and Rose, 1967). Synthesis of unsaturated fatty acids is favoured when the growth temperature is lowered. The same effect is produced by increasing the dissolved oxygen tension (Jollow et al., 1968). It is probable that these two parameters are interrelated since the dissolved oxygen tension increases at lower temperatures.

Presporulation cells are normally incubated at 30°C and transferred to sporulation medium at 25°C. Cells grown in presporulation medium at 25°C form 5-10% fewer asci than cells grown at 30°C. The degree of unsaturation of fatty acids in lipids from cells grown at 25°C (68%) is considerably less than the degree of unsaturation of fatty acids from asci at T_{24} (78%).

Acid	Period in sporulation medium at 25°C (h)			
	0	24	120	0
12:0	3.47 ± 0.77	0.56 ± 0.19	tr	0.77 ± 0.32
13:1	0.87 ± 0.02	tr	tr	tr
14:0	1.48 ± 0.29	1.14 ± 0.70	1.03 ± 0.02	1.29 ± 0.40
15:0	1.04 ± 0.08	0.60 ± 0.04	0.74 ± 0.09	tr
16:0	25.74 ± 2.98	15.57 ± 0.75	8.43 ± 0.73	23.03 ± 2.04
16:1	39.00 ± 0.93	44.24 ± 1.80	50.73 ± 1.68	31.40 ± 2.34
18:0	4.76 ± 1.02	4.95 ± 0.44	3.13 ± 0.86	6.60 ± 0 24
18:1	23.83 ± 2.16	33.32 ± 0.96	35.45 ± 0.58	36.71 ± 3.38
% saturated acids	35.45	23.09	13.70	31.89
% unsaturated acids	63.69	77.90	86.18	68.11

Table 2. Fatty acid composition of lipids of Saccharomyces cerevisiae during ascus formation

Values quoted are the molar percentages of the total fatty acid with 95% confidence intervals. Values in the far right hand column refer to cells grown at 25°C instead of 30°C. tr indicates that the percentage of fatty acid was less than 0.5%. The following fatty acids were also present at less than 0.5%: 8:0, 9:0, 10:0 and 13:0.

Sterol composition of developing asci. Examination of thin-layer chromatograms showed that two forms of sterol are present in lipid extract of cells and developing asci, namely free sterol and esterified sterol. Figs. 6 and 8 show that, of the total sterol present in cells, about 50% occurs in the free form, but as sporulation proceeds this value decreases to only 23% at T_{120}.

It has been reported (Hunter and Rose, 1972) that recovery of sterols from Sacch. cerevisiae is increased by about 24% if whole cells are subjected to saponification followed by acid hydrolysis, rather than extracted with chloroform-methanol. Sterols in both of these extracts were assayed using the Liebermann-Burchard method (Moore and Baumann, 1952) and the Shaw and Jefferies (1953) method. Both methods assay conjugated diene sterols, (for example, ergosterol). However the Liebermann-Burchard method distinguishes between two types of sterol, fast-reacting sterol and slow-reacting sterol. Fast-reacting sterols are the conjugated diene type; slow reacting sterols (for example, cholesterol) do not have a conjugated diene structure. The amounts of sterol assayed by the Liebermann-Burchard method and by that of Shaw and Jefferies at T_0 and T_{120} do not show statistically significant differences. The amounts of sterol assayed at T_{24}, however, do show a small significant difference (see Fig. 11). At T_0 saponification followed by hydrolysis extracts 20-60% more sterol than chloroform-methanol, but at T_{24} the amounts are equal and at T_{120} 30% more sterol is extracted by chloroform-methanol, although this value is not statistically significant.

Analysis of sterols by gas-liquid chromatography revealed two major components and trace amounts of two others (Table 3). One of the major components has a relative retention time of 2.52, relative to

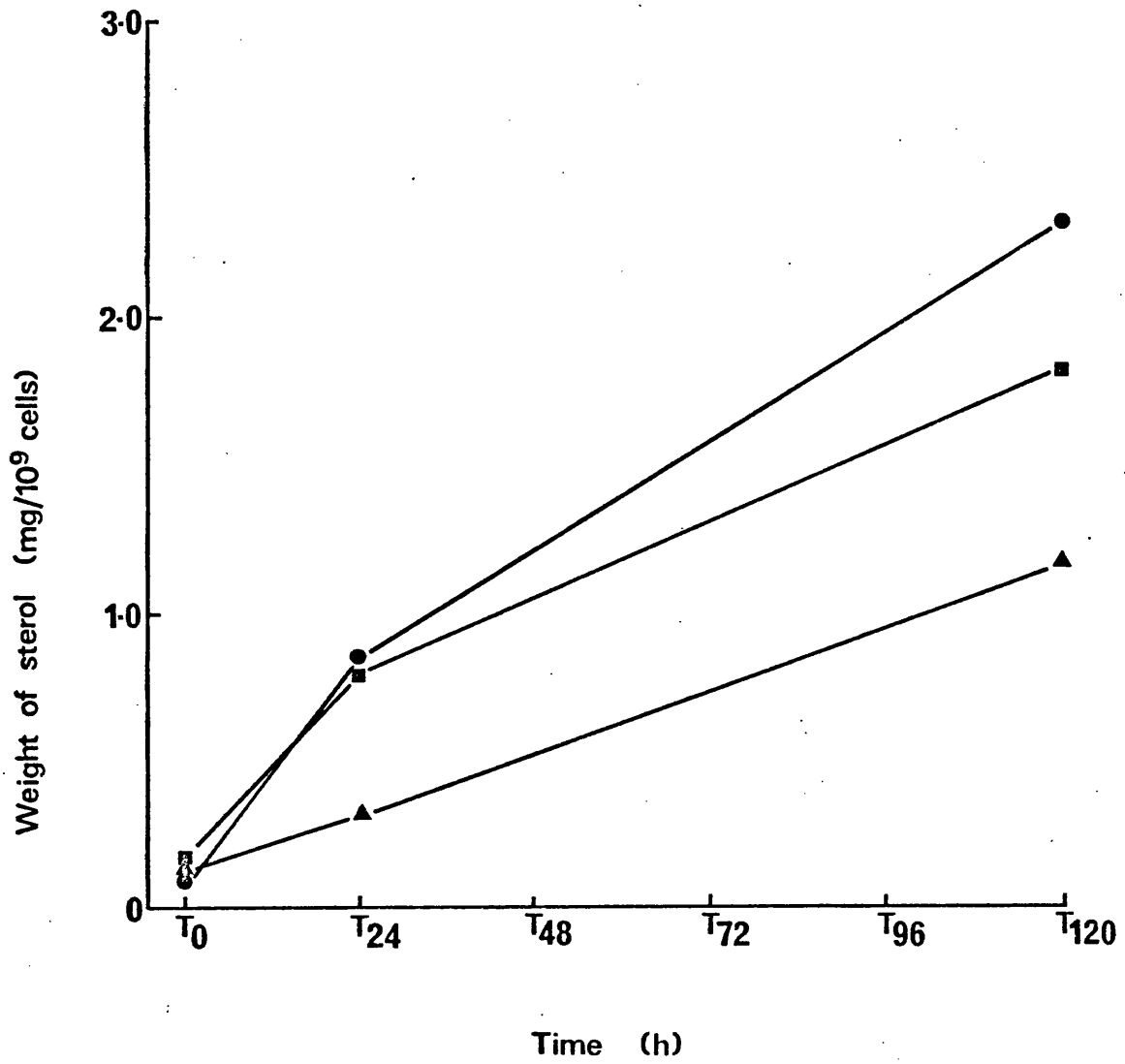


Figure 11. Total sterol content of developing asci of Saccharomyces cerevisiae.

■——■ sterols prepared by saponification of whole cells (see Methods) followed by assay by the Liebermann-Burchard method.

▲——▲ sterols prepared by saponification of whole cells (see Methods) followed by assay by the Shaw and Jefferies method.

●——● sterols in the chloroform-methanol extract assayed by the Liebermann-Burchard method,

The SEM value of each point was less than 15%.

Retention time of sterol relative to cholestane	Sterols extracted from cells using	Sterol content of cells incubated in sporulation medium (%)		
		0h	24h	120h
1.70	$CHCl_3$-MeOH	tr	tr	tr
1.95	$CHCl_3$-MeOH	tr	tr	tr
2.52	$CHCl_3$-MeOH	62.0±7.2	66.7±3.0	60.6±5.3
	saponification	72.5±6.5	63.9±5.8	63.7±8.4
3.00	$CHCl_3$-MeOH	35.2±8.6	33.3±3.0	39.4±5.4
	saponification	27.8±6.6	34.4±4.8	36.3±8.4

Table 3. Gas-liquid chromatographic determination of proportions of individual sterols (free and esterified) in developing asci of Saccharomyces cerevisiae.

Sterols were separated by gas-liquid chromatography using a column of OV-17.

Values quoted are percentage of the total sterol with 95% confidence limits. tr indicates that the percentage of sterol was less than 0.5% of the total.

cholestane, which is identical with the relative retention time of ergosterol, or its appropriate derivative, and was tentatively identified as such. The other component, which is present in amounts equal to about half that of ergosterol, was at first tentatively identified by its relative retention time of 3.00 as the tetraethenoid sterol described in Sacch. cerevisiae NCYC 366 by Longley, Rose and Knights (1968) and Hunter and Rose (1972). However this sterol did not exhibit the absorption maximum at 233nm reported by Longley et al. (1968) and I was therefore unable to identify it. The proportions of the two major sterols did not differ very much as sporulation proceeded; neither did the extraction procedure affect the proportions of the two sterols.

Incorporation of acetate into lipids of developing asci. Metabolism of acetate and lipid synthesis were studied using sodium [U- ^{14}C] acetate. Presporulation cells were introduced into sporulation medium which contained the labelled nutrient. Incorporation of acetate carbon by cells and lipids was followed; the results are shown in Figs. 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12. The incorporation of acetate carbon by squalene was not investigated.

When [U- ^{14}C] acetate was present in sporulation medium incorporation of the isotope into cells (Fig. 12) proceeded in a manner which followed closely the increase in dry weight of heat-dried asci (Fig. 1). Incorporation of acetate proceeds linearly from T_0 to T_{30}, after which time there is no further net increase in acetate incorporated; the ascan content remains constant up to T_{120}. Incorporation of acetate into total lipids during sporulation proceeds rapidly from T_0 to T_{12} and then continues more slowly up to T_{120} (Fig. 3). About 50% of the

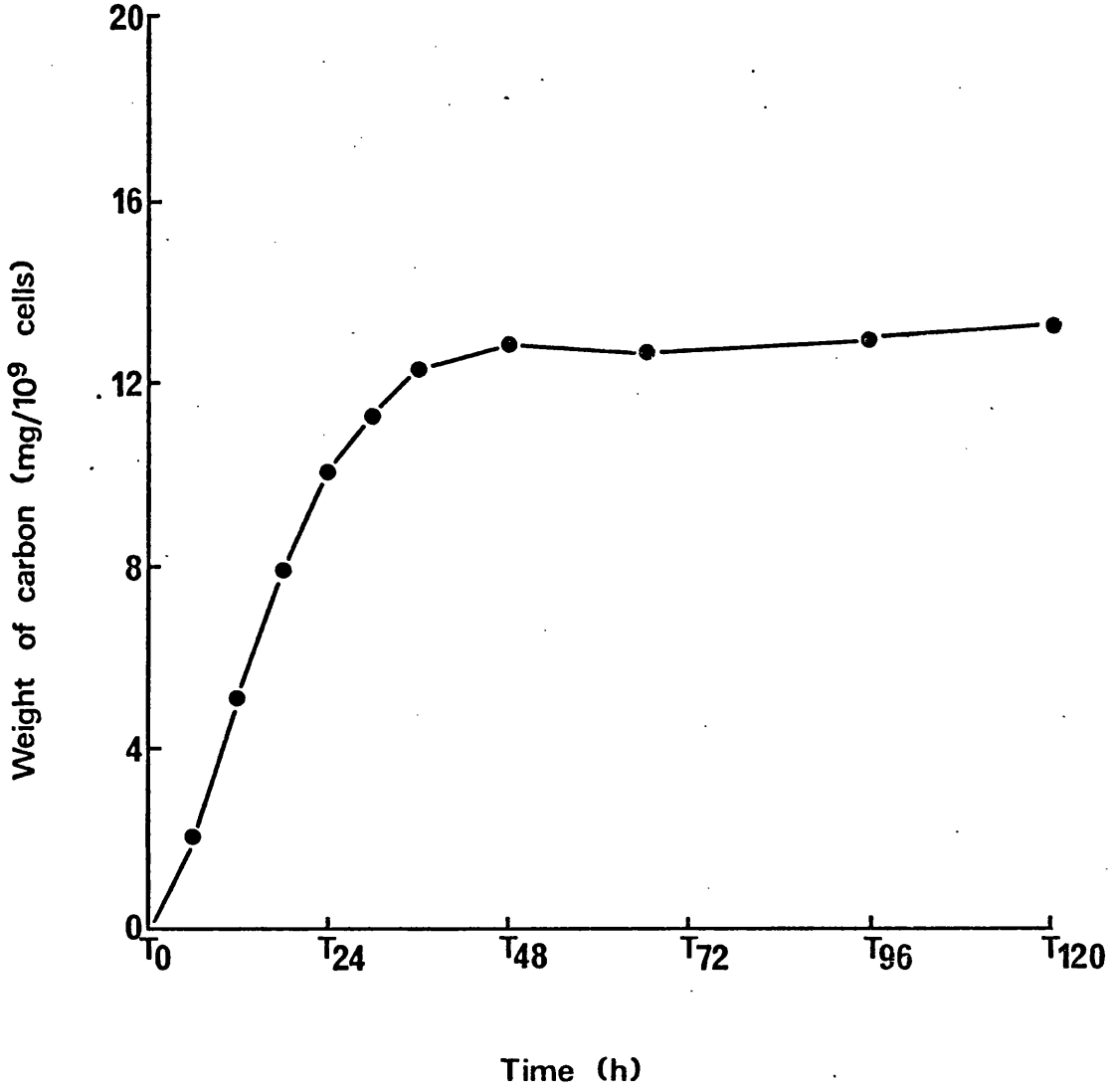


Figure 12. Incorporation of acetate carbon into developing asci of Saccharomyces cerevisiae when $\left(U-{}^{14}C\right)$ acetate (50μCi/Mole) was present in the sporulation medium.

The SEM value of each point was less than 2%.

acetate carbon incorporated into asci was recovered in the lipid fraction at T_{120} (compare Figs. 3 and 11). Esposito et al. (1969) reported that 10% of potassium [2 -^{14}C] acetate incorporated into asci was recovered from the lipids at T_{50}. The pattern of incorporation of acetate carbon into total ascan lipids is reflected in the incorporation carbon into phospholipids, sterol esters and triacylglycerols (Figs. 4, 5, 6). Sterol esters are so rapidly labelled that, at T_{120}, virtually 100% of the increase in weight of this lipid class is accounted for by acetate carbon (Fig 5). Phospholipids and triacylglycerols are less rapidly labelled, and at T_{120} only 30% and 50% respectively are labelled with acetate carbon (Figs. 4, 5).

Incorporation of acetate carbon into sterols was very similar to that reported for sterol esters (compare Figs. 6 and 8). The pattern of incorporation of acetate carbon into the other two minor fractions was more erratic (Figs. 8, 9).

Lipid synthesis during ascus development. The rate of lipid synthesis was measured during ascus formation by pulse-labelling sporulating cells with sodium [U- ^{14}C] acetate during the first 42h and the last 6h of sporulation. Labelled acetate was added at intervals to suspensions of sporulating cells and, after a further 6h incubation, the asci were harvested. The incorporation of acetate carbon by cells and lipids was measured after each 6h period. The results of these experiments are shown in Figs. 12, 13, 14, 15 and 16. The rate of incorporation of acetate carbon into asci and lipids (Fig. 12) indicates that there are two periods of maximum acetate incorporation. These were recorded between T_6 and T_{18}, and between T_{24} and T_{30}. The individual lipid fractions also exhibit a marked peak of incorporation of acetate during

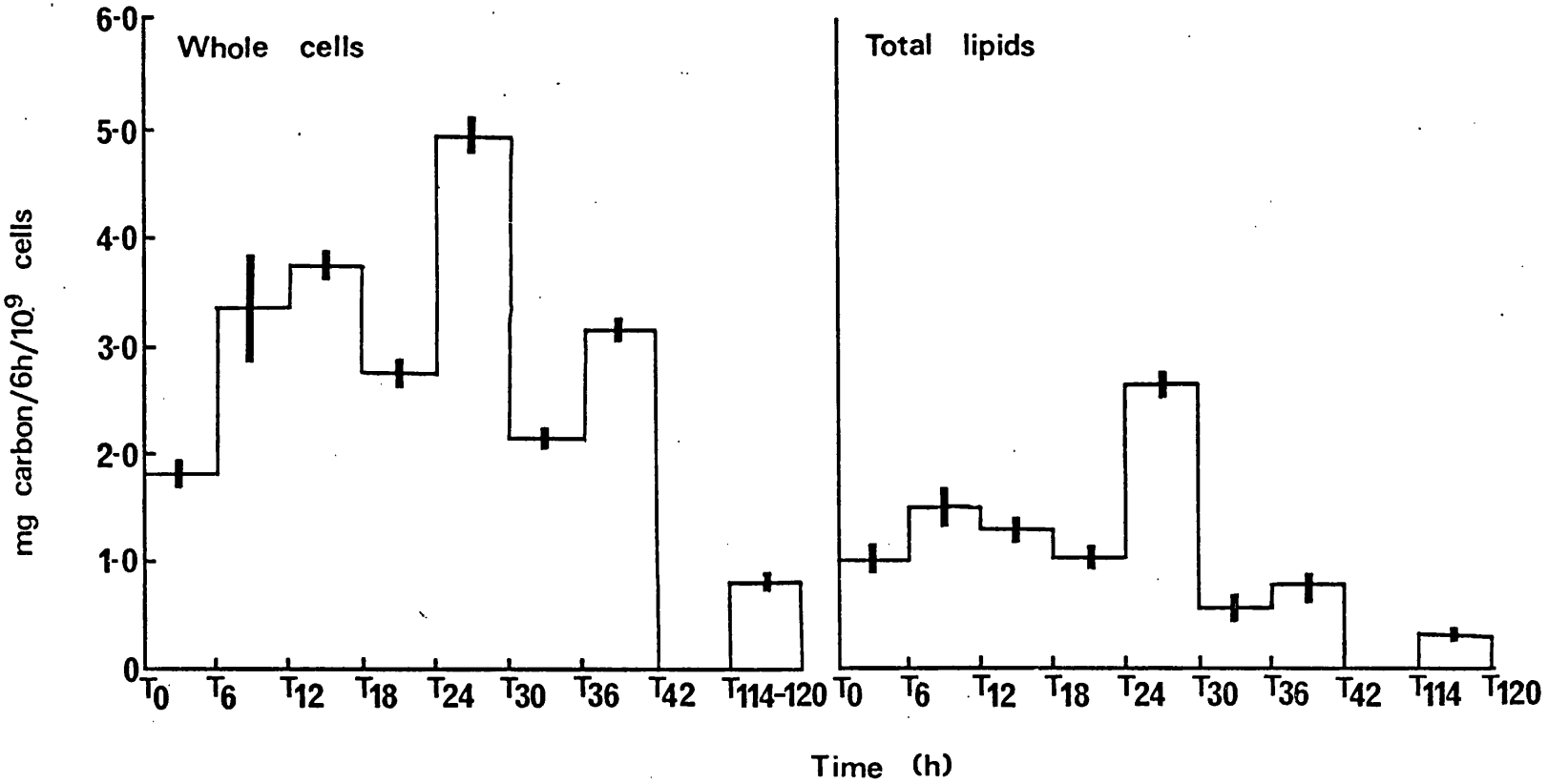


Figure 13. Pulse-labelling of whole cells and total lipids of Saccharomyces cerevisiae following addition of $(U\text{-}^{14}C)$ acetate to sporulating cultures. Labelled acetate (50μCi/Mole) was added to sporulating cultures at intervals and incorporation of isotope was followed over six hour periods. Blocks represent the average incorporation of acetate carbon during a six hour pulse; bars represent 95% confidence intervals.

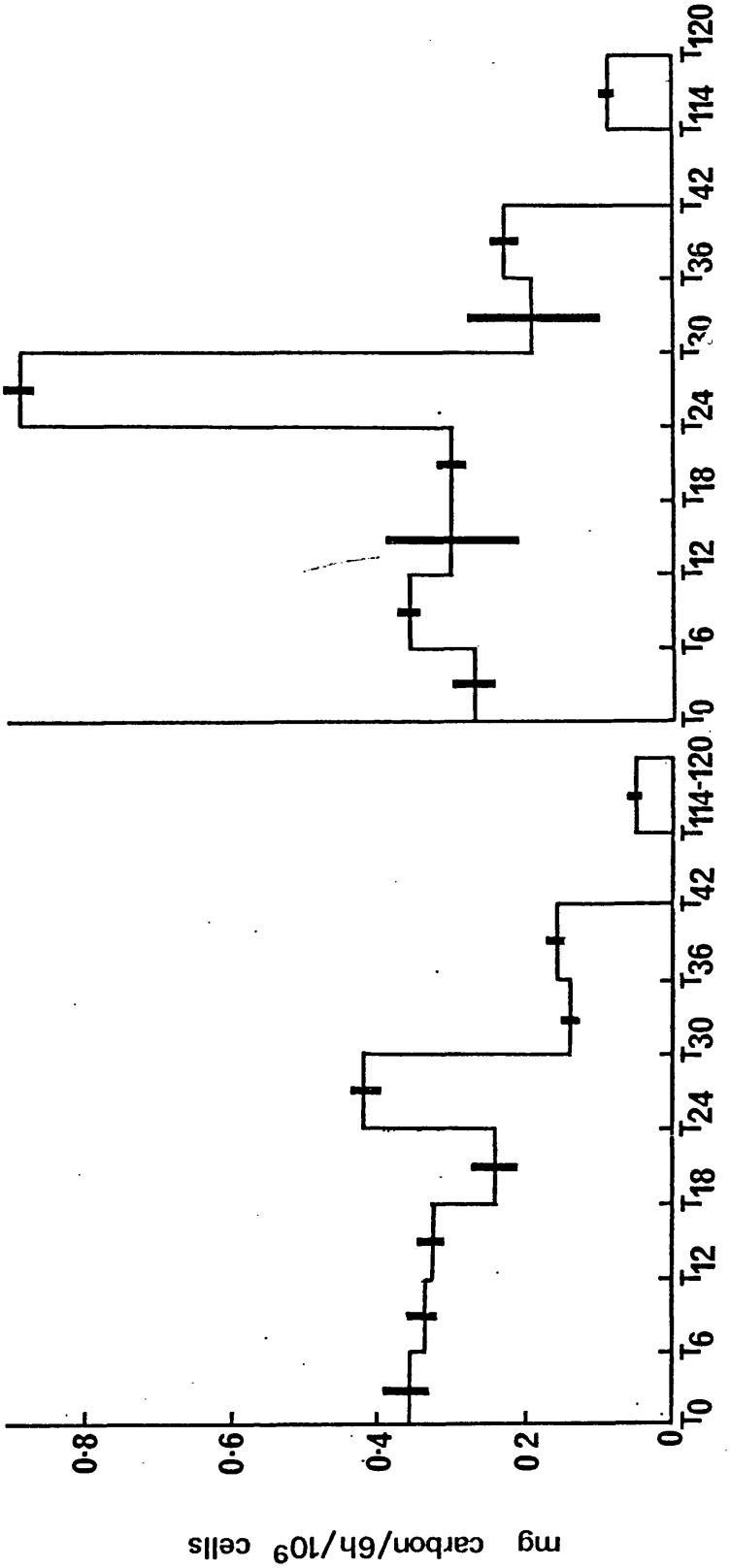


Figure 14. Pulse-labelling of phospholipids and sterol esters of Saccharomyces cerevisiae following addition of $(U\text{-}^{14}C)$ acetate to sporulating cultures. Labelled acetate 50μCi/Mole) was added to sporulating cultures at intervals and the incorporation of isotope was followed over six hour periods. Blocks represent the average incorporation of acetate carbon during a six hour pulse; bars represent 95% confidence intervals.

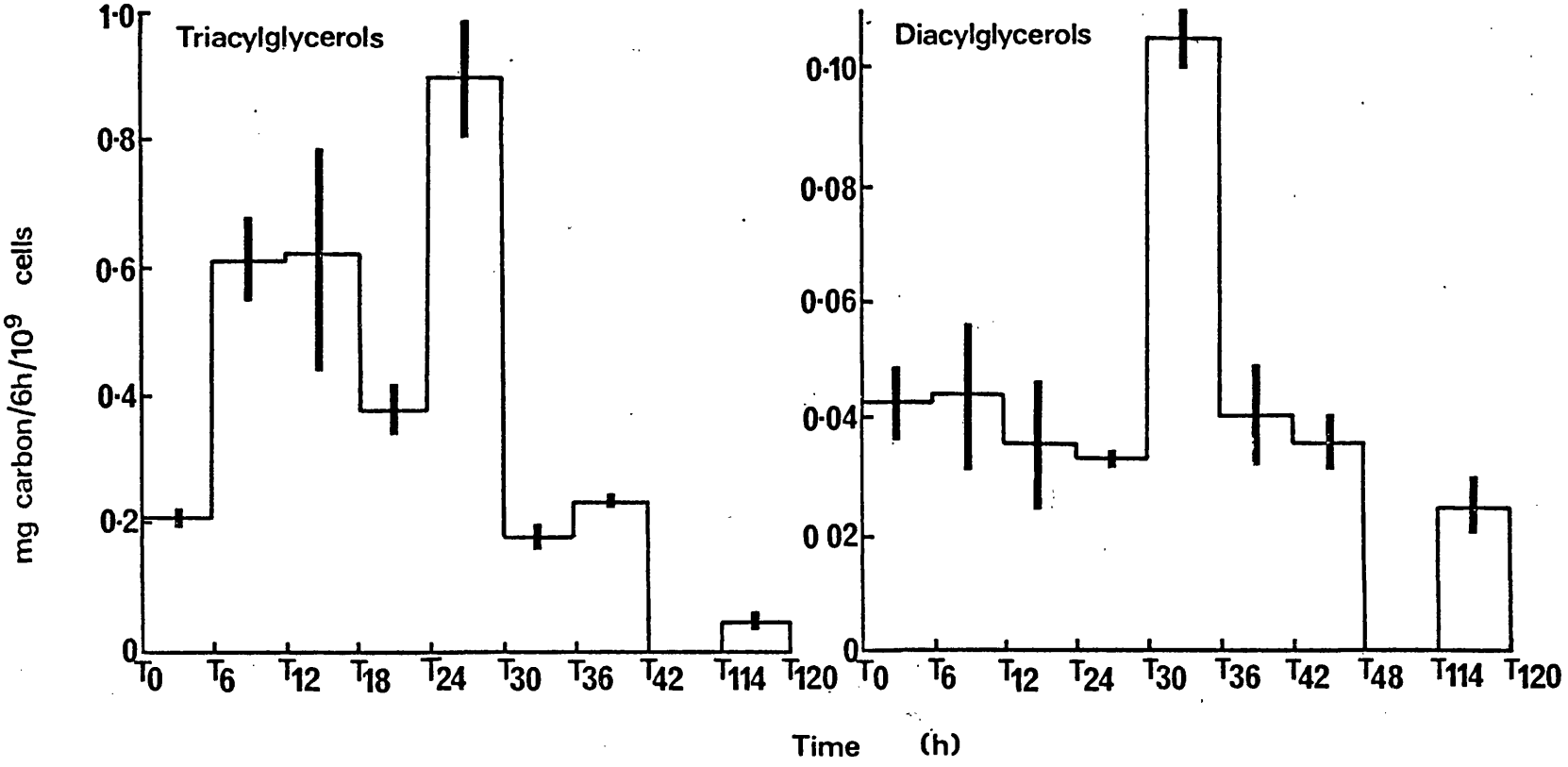


Figure 15 Pulse-labelling of triacylglycerols and diacylglycerols of Saccharomyces cerevisiae following addition of $(U-{}^{14}C)$ acetate to sporulating cultures. Labelled acetate (50μCi/Mole) was added to sporulating cultures at intervals and the incorporation of isotope was measured over six hour periods. Blocks represent the average incorporation of acetate carbon during a six hour pulse; bars represent 95% confidence intervals.

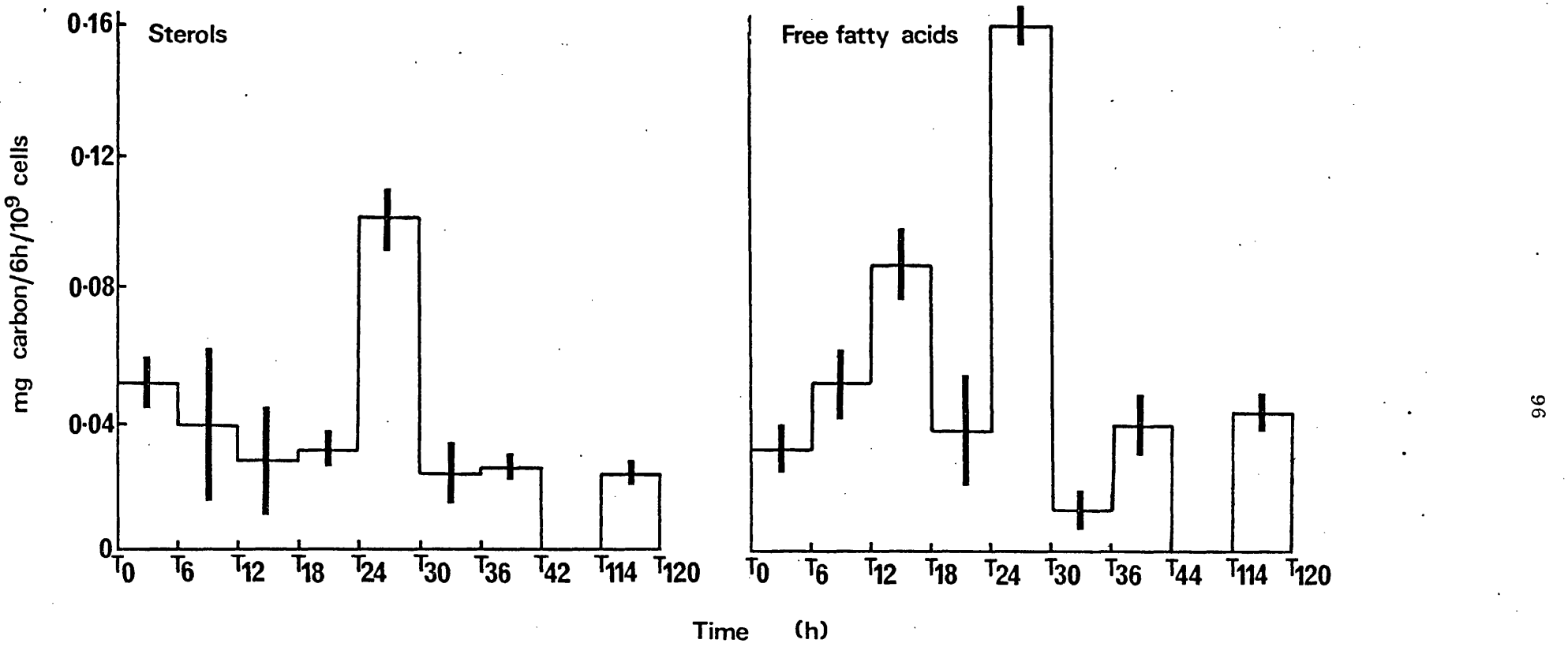


Figure 16. Pulse labelling of sterols and fatty acids of Saccharomyces cerevisiae following addition of $(U\text{-}^{14}C)$ acetate to sporulating cultures. Labelled acetate (50μCi/Mole) was added to sporulating cultures at intervals and the incorporation of isotope was measured over a six hour period. Blocks represent the average incorporation of acetate carbon during a six-hour pulse; bars represent 95% confidence intervals.

the second period, namely between T_{24} and T_{30} (Figs. 14, 15, 16). However not all lipids exhibited the first peak of activity (between T_6 and T_{18}). Triacylglycerols and free fatty acids exhibited a peak of activity between T_6 and T_{18} (Figs. 15, 16) and phospholipids and sterols showed a broader based peak of activity between T_0 and T_8 (Figs. 14, 16).

INHIBITION OF ASCUS DEVELOPMENT BY AMMONIUM IONS.

Nitrogenous compounds (for example ammonium salts and amino acids) when incorporated into sporulation media which include carbon-containing substrates such as glucose or acetate, prevent or delay sporulation in yeasts. Fig. 17 shows the effect of different concentrations of ammonium sulphate on the degree of sporulation in Sacch. cerevisiae. Control experiments were performed using sodium sulphate in place of ammonium sulphate and showed that inhibition was due to the presence of ammonium ions rather than sulphate ions.

Ammonium sulphate was added to cultures of sporulating cells after various periods of incubation in order to measure the duration of sensitivity to this inhibitor. The results of this experiment are shown in Fig. 18. Sporulating cells are sensitive to inhibition by ammonium ions up to T_{24} in sporulation medium. After this time cultures become insensitive to ammonium ions.

Figure 18 shows that the cells are sensitive to inhibition by ammonium ions during the first 24h of sporulation. The following experiment was carried out in order to find out if cells which had been incubated

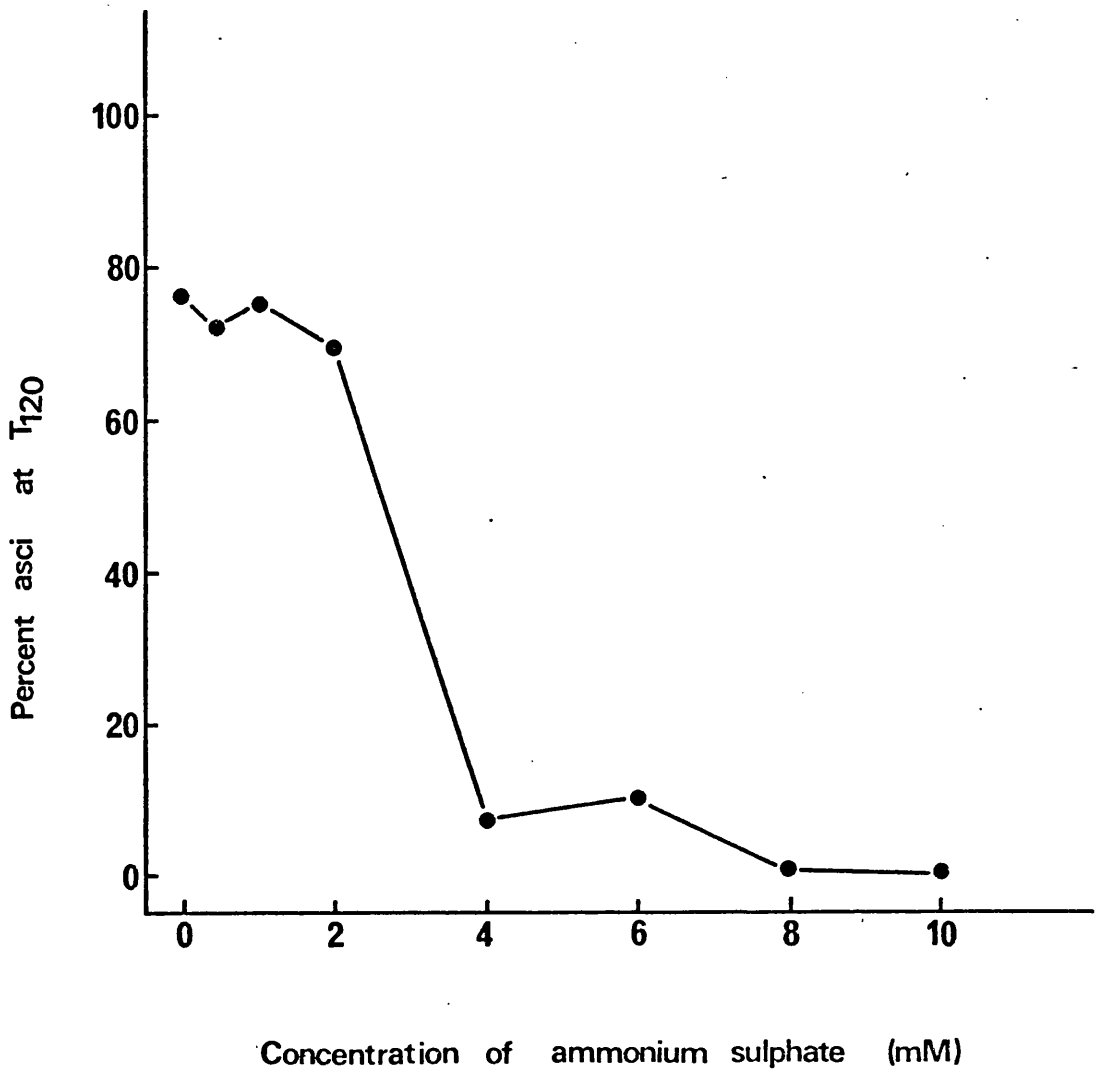


Figure 17. Inhibition of ascus formation by ammonium sulphate. Ammonium sulphate was added to sporulating cultures, to a final concentration as indicated, at T_o. The percentage of cells forming asci was determined at T_{120} for each concentration of ammonium sulphate.

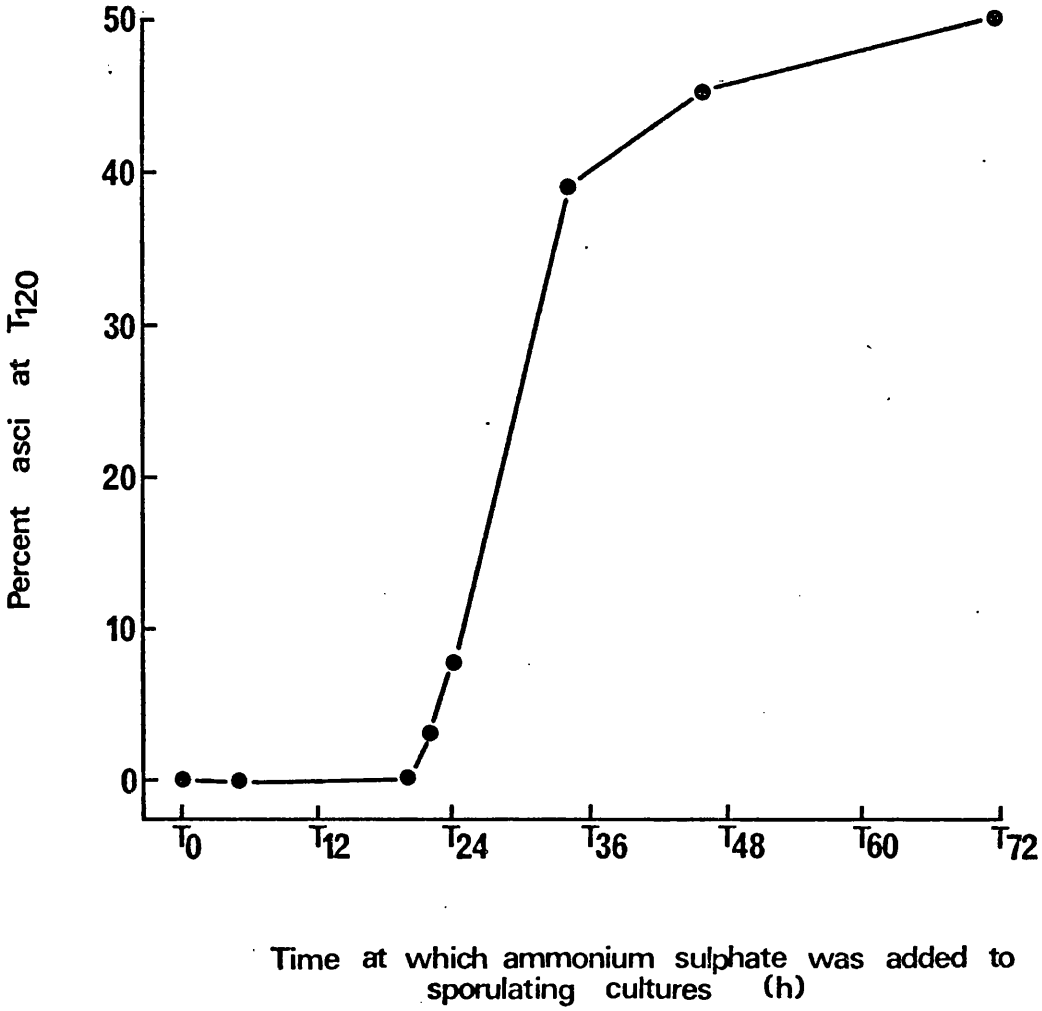


Figure 18. Inhibition of ascus formation by ammonium sulphate. Ammonium sulphate was added to sporulating cultures to a final concentration of 10mM at the times shown. The The percentage of cells forming ascospores at T_{120} was determined.

in the presence of ammonium sulphate during the early stages of sporulation, but later removed from such a medium, were able to sporulate. Cells were incubated in sporulation medium which contained ammonium sulphate. At intervals thereafter cells were removed from this medium by centrifugation and resuspended in fresh medium which was free of ammonium sulphate. The percentage of cells which formed asci was counted at T_{120}. The results of this experiment are summarised in Fig. 19. It is clear that the presence of ammonium ions did not interfere with the formation of asci while the inhibitor was present between T_0 and T_{20}. When the inhibitor is present from T_0 to T_{30} then formation of asci is inhibited.

The results summarised in Figs. 18 and 19 suggest that the most sensitive period to inhibition by ammonium ions extends from T_{20} to T_{30}. Ammonium sulphate was added to sporulating cultures to test this assumption; the ammonium ions were added at various times throughout the course of sporulation. Six hours later the cells were removed from this medium by centrifugation and resuspended in fresh sporulation medium which did not contain ammonium sulphate. The percentage of cells forming asci was counted after 120h. The results of this experiment are shown in Fig. 20. Sporulation is most sensitive to inhibition by ammonium ions in the period T_{24} to T_{30} as expected. The presence of ammonium sulphate outside this period does not appear to affect ascus formation.

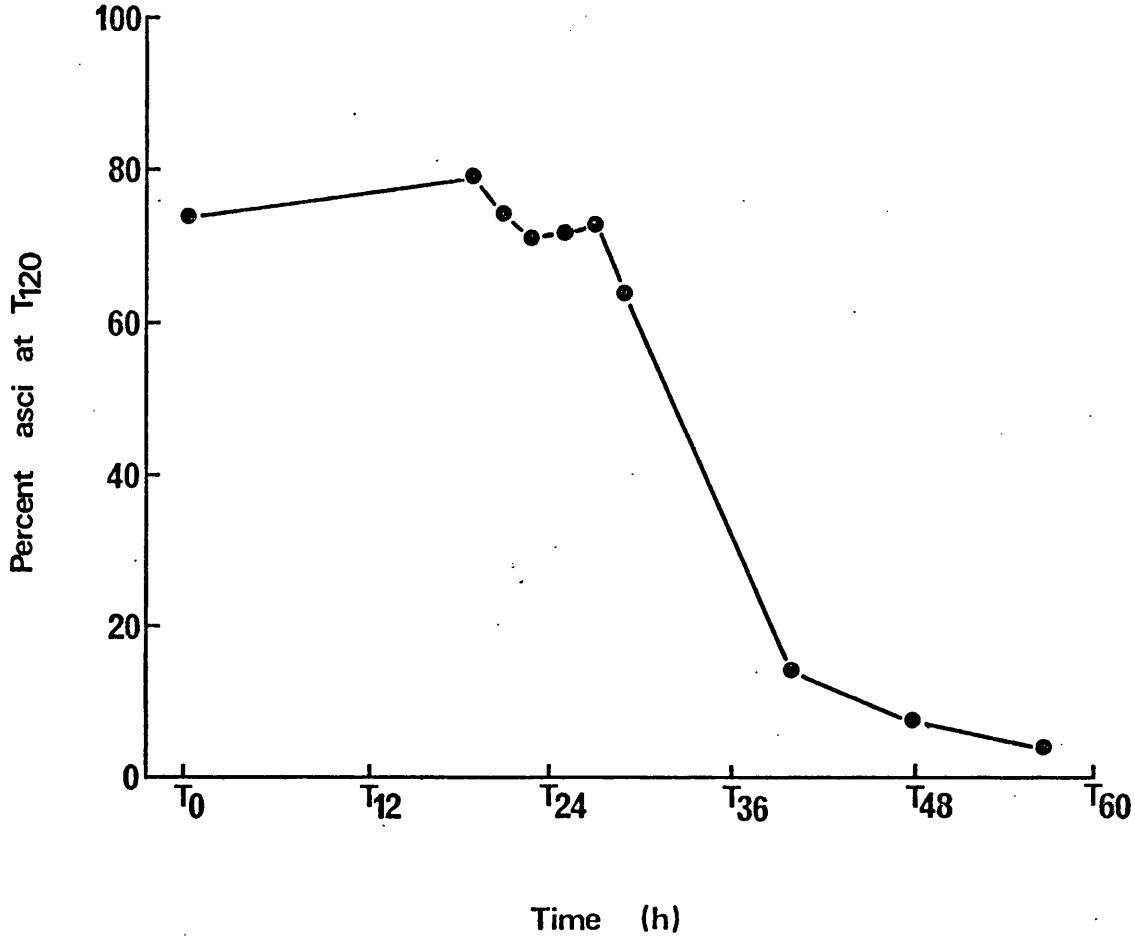


Figure 19. Inhibition of ascus formation by ammonium sulphate. Ammonium sulphate was added to sporulating cultures, to a final concentration of 10mM, at T_0. At intervals sporulating cells were removed from this medium and resuspended in fresh sporulation medium and left to incubate up to T_{120}. The percentage of cells forming ascospores at T_{120} is plotted on the ordinate, time of removal of ammonium sulphate from the medium is plotted on the abcissa.

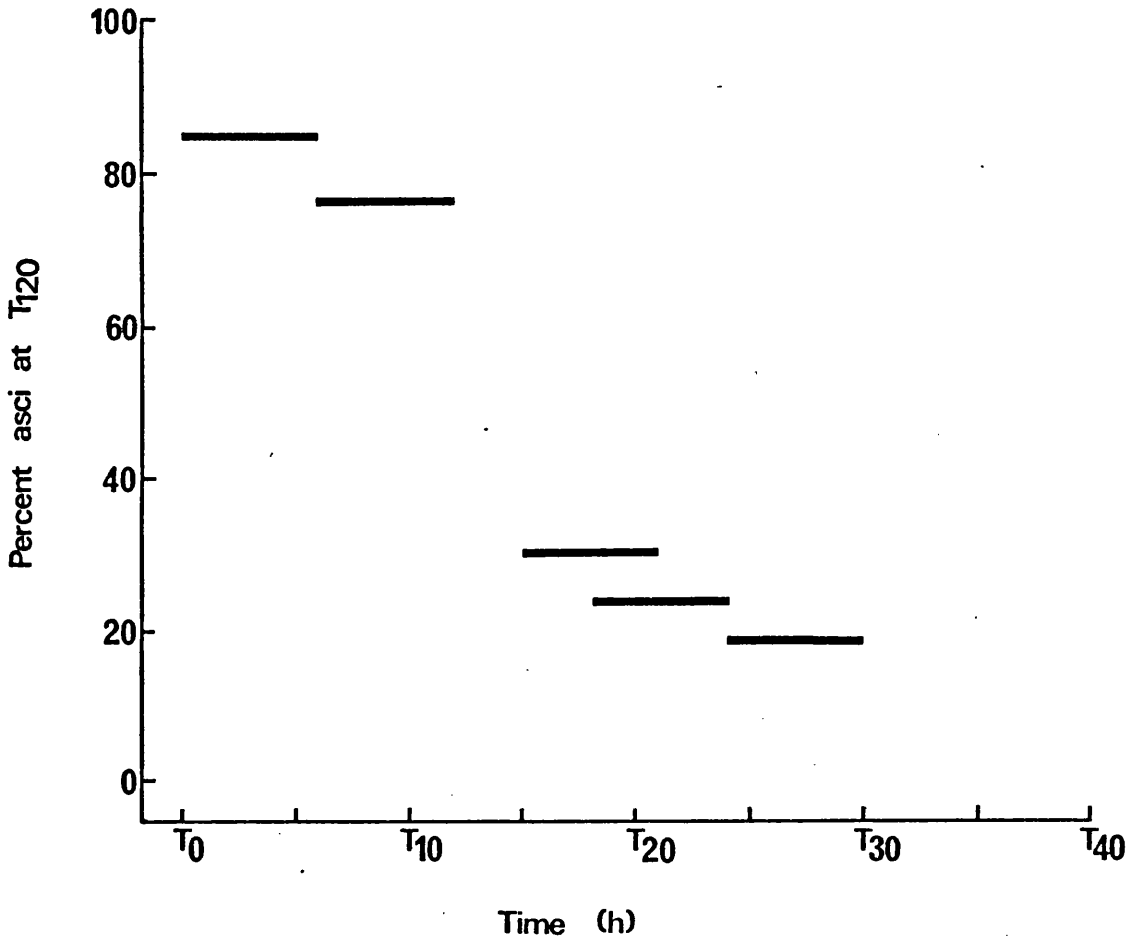


Figure 20. Inhibition of ascus formation by short periods of exposure to ammonium sulphate. Ammonium sulphate, to a final concentration of 10mM, was added to sporulating cultures at intervals. After a further incubation of six hours the sporulating cells were removed from this medium and suspended in fresh sporulation medium and left to incubate up to T_{120}. The percentage of cells forming ascospores at T_{120} is plotted on the ordinate; period of exposure to ammonium sulphate is plotted on the abcissa.

INVESTIGATION INTO THE CHEMICAL NATURE OF THE ASCOSPORE SURFACE

The surfaces of ascospores from Sacch. cerevisiae are known to be hydrophobic (Emeis, 1958) and stain deeply with Sudan Black B (Miller and Hoffmann-Ostenhof, 1964). Consequently it has been suggested that the outermost layer is composed of lipid (Langeron and Luteraan, 1947; Emeis, 1958; Miller and Hoffmann-Ostenhof, 1964) although this suggestion is not consistent with the marked ultra-violet-absorbing properties of this layer (Miller et al., 1963). Mundkur (1961a, b) believes that the spore wall contains large amounts of acetylglucosamine, a belief which is supported by the observation that the spore walls stain deeply with gallocyanin. The spore-wall surface is serologically distinct from the cell wall surface (Miller and Hoffmann-Ostenhof, 1964). Spore walls are more resistant to an enzyme mixture prepared from the crop of the Roman snail, Helix pomatia (Johnson and Mortimer, 1959) and pronase (Rousseau and Halvorson, 1969) than are vegetative cell walls.

It has been shown that changes in the electrophoretic mobilities of microorganisms with pH value measured in buffers of constant ionic strength, can be interpreted in terms of the ionogenic groups present on the cell surface (Richmond and Fisher, 1973). In view of this and earlier investigations (Dyar and Ordal. 1946; Dyar, 1948), the shape of the pH-mobility curve of untreated ascospores from Sacch. cerevisiae (Fig. 21) suggests the presence of amino and carboxyl groups on the surface.

Electrophoretic measurements in the presence of sodium dodecyl sulphate (SDS) showed that lipid was probably not situated on the surface of the ascospore. The mobility of ascospores was not affected by the

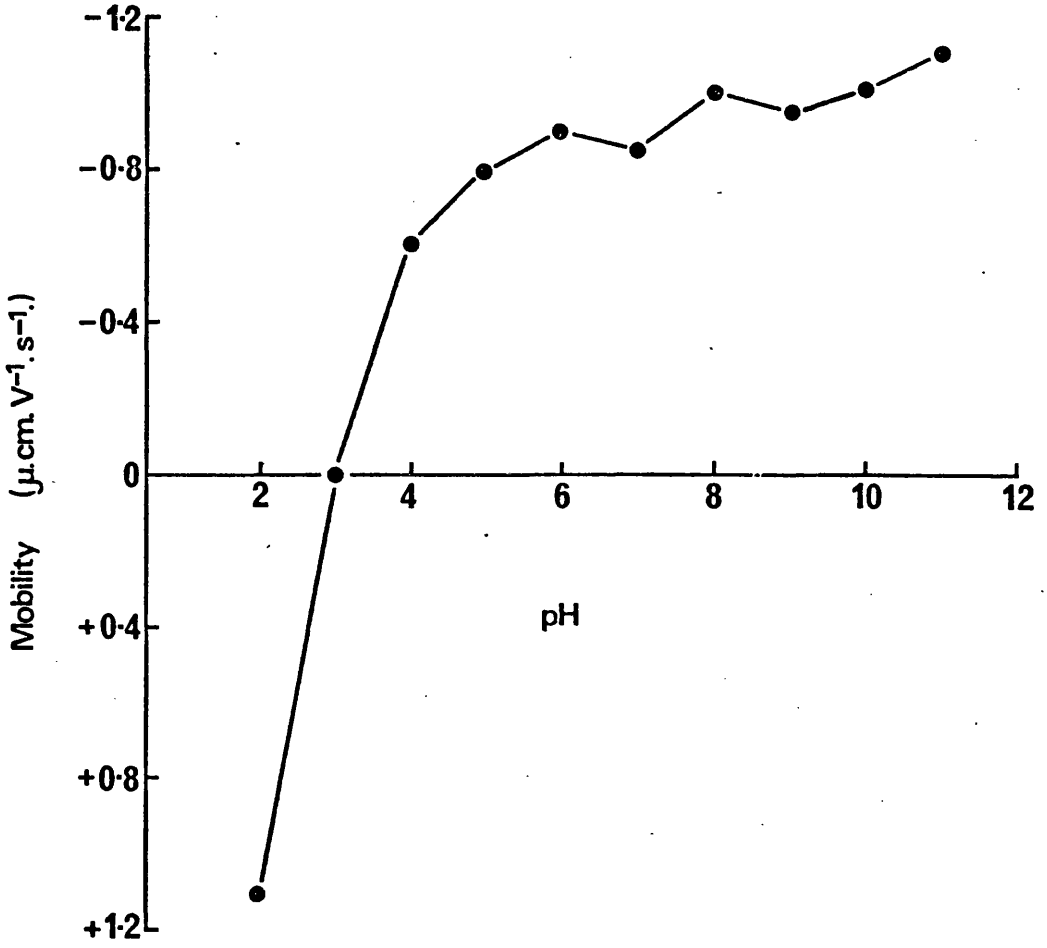


Figure 21. Effect of the buffer pH value on electrophoretic mobility of ascospores from Saccharomyces cerevisiae. (see Methods)

presence of 1μM, 10μM and 100μM SDS respectively, in 10mM phosphate buffer (pH 7.0). Sodium dodecyl sulphate, which is an anionic detergent, produces changes in electrophoretic mobility which can be related to presence of surface lipid, at concentrations lower than those required to cause lysis. Dyar (1948) found that an emulsion of lipid droplets suspended in SDS became more negative with increasing concentrations of the detergent. On the other hand protein (for example, egg albumen) and carbohydrates (for example, cellulose) possess a constant mobility in solutions of SDS of different concentrations.

Subsequent investigations into the nature of the ascospore surface were carried out in this laboratory by Mr M Briley. This involved the digestion of the ascospore surface by enzyme preparations which included trypsin, chymotrypsin and pepsin, and treatment of the ascospores with 8M urea. These experiments provided further evidence for the presence of a surface protein layer on ascospores from Sacch. cerevisiae DCL 740. This work has been reported elsewhere (Briley, Illingworth, Rose and Fisher, 1970).

DISCUSSION

The work reported in this thesis correlates modifications in fine structure with the changes in the lipid composition that occur in Saccharomyces cerevisiae during ascosporogenesis. Also included are data on the inhibition of sporogenesis by ammonium ions. These two sets of data are discussed separately.

CORRELATION BETWEEN FINE STRUCTURE AND LIPID COMPOSITION

Information gained from studies of cytological changes observed in conjunction with physiological and biochemical activities is clearly of especial value to cell biologists. Cellular differentiation is a many-faceted process, the examination of which requires the talents of biologists working in several disciplines.. The specialist researching in one very closely defined area may well miss or ignore points of very real importance which happen to lie outside his or her field of knowledge. It is particularily true that an appreciation of the mechanisms involved in a differentiation system such as sporulation in yeast requires the combined skills of cytologists, physiologists, biochemists and geneticists. When reviewing the literature concerned with sporulation in yeast it is possible to collate all the various aspects of the process from the work of individual researchers. Unfortunately, in the past, simultaneous studies of different aspects of sporogenesis have been performed all too infrequently. For example workers have studied changes in composition during sporogenesis (Esposito et al., 1969; Roth, 1970) without attempting to relate these changes to alterations in fine structure. However there are exceptions; Pontefract and Miller (1962) related the disappearance of fat globules in sporulating cells with the development of spore walls. Later, Croes (1967a) and Sando and Miyake (1971a), employing Giemsa

staining, correlated nuclear events during meiosis with a number of biochemical and physiological events including increased cell weight, duplication of DNA and increased contents of RNA and protein.

Lipids occur mainly in two types of organelle in Saccharomyces cerevisiae, namely in lipid vesicles and in plasma and intracellular membranes. While virtually nothing is known of the structure or function of vesicles, a certain amount of information has been collected concerning the composition, structure and function of yeast membranes. However biologists are still fairly ignorant of the precise roles of the various types of lipid in membranes. The yeast plasma membrane contains large amounts of lipid, mainly in the form of phospholipid and sterol (Hunter and Rose, 1971), and its structure has been well reviewed by Matile, Moor and Robinow (1969) and Matile (1970). The yeast plasma membrane can be considered to have three main functions. The first is to form a barrier between the protoplast and its environment; the second to act as an organelle which controls the uptake and secretion of solutes; and the third to serve as an organelle on which the components of the cell wall are synthetised. An excellent review of the role of the yeast plasma membrane is given by Suomalainen and Oura (1971). Except for mitochondrial membranes (Hunter and Rose, 1971), much less is known about the function, composition and structure of other membranes which are known to exist in the yeast cell. These membranes have been discussed by Matile et al. (1969).

In experiments designed to analyse the lipid composition of sporulating yeast cells several problems had to be faced. Firstly the inherent difficulties of separating asci from non-sporulating cells are obvious

and no such separation was attempted. One can only presume that the metabolism of viable non-sporulating cells is different from that of developing asci. Since it is not possible to separate the effects of the two metabolic processes it has been assumed that the gross changes measured during sporogenesis are a result of the activity of developing asci and that the contribution to these changes by non-sporulating cells is minimal. In any case the high degree of sporulation, (between 80% and 90%) exhibited by the strain of Saccharomyces cerevisiae used in this study helps to alleviate this problem. This degree of sporulation is considerably greater than that (between 60% and 70%) observed by Croes(1967a) and Esposito et al. (1969).

Secondly, it was not found possible to isolate significant amounts of spores from asci. Several methods for isolating populations of yeast ascospores were investigated in a series of preliminary experiments. These included a method (Sacks and Alderton, 1961) involving flotation of spores, which had been released from asci, in paraffin oil (clearly a dubious method in view of later lipid analyses) and separation of spores from ascan debris and non-sporulated cells by electrophoresis (Resnick, Tippetts and Mortimer, 1967). The latter process produced insufficient quantities of spores for analytical purposes. Analyses performed on mature asci therefore measured the combined composition of spores and epiplasm. This second problem is less acute when examining asci which are in the process of development since it is important to know how whole cells are changing as a result of sporogenesis.

When studying any developmental system, such as sporulation in yeast, it is necessary to distinguish between temporal development and spatial development. Temporal development is a sequence of events,

ordered in time, which is set in motion by certain environmental changes. In yeast sporogenesis the change of environment is from a relatively complete presporulation medium to a sporulation medium of near starvation conditions. The nature of the 'trigger' which initiates this programme of sequential events is as yet unknown. In the light of existing knowledge of metabolic control it is probable that these events are ordered by a programme of sequential gene de-repression. It is likely however that several different sequences of de-repression operate during sporogenesis and the existence of a biological 'clock', or indeed several 'clocks' may be responsible for the correct timing sequence of such trains.

Parallel with temporal development is development in space. Spatial development during differentiation operates at two levels, namely inter-cellularly and intra-cellularly. In the former some cells undergo temporal differentiation and others do not, so that one can have one or more types of cell existing together in one multicellular organism (for example, heterocyst formation in blue-green algae). In intra-cellular differentiation various components within the protoplasm remain spatially distinct during development. For example, in yeast ascosporogenesis certain components are produced at one site in the cell and are later transported to another site where they can be incorporated into the spore. Other components are elaborated at the site at which they are incorporated into the spore.

In view of the importance of the temporal sequence of events in yeast sporulation I propose to discuss changes in lipid composition and fine structure in the order in which they occur in time. I shall take arbitrary periods within the sporulation sequence and discuss the changes that occur in these periods. Spatial development events will

be discussed as they happen.

Period 1: T_0 to T_{12} Although the Croes' convention for describing the timing of events during sporulation has been adopted in this thesis this does not imply that events in the present study are synchronous with Croes' observations. The timing of events in sporogenesis varies widely between cells of different strains as indeed it does between cells in the same culture. For example, Croes (1967a) found that cells exhibiting meiotic configurations were most abundant between T_{12} and T_{16} whereas Sando and Miyake (1971) observed meiotic configurations between T_8 and T_{10}. In the present study nuclear changes are most obvious between T_{18} and T_{24}. According to Croes (1967a) ascospores are visible after T_{18}; Esposito et al. (1969) were able to see ascospores after T_{15}; Sando and Miyake (1971) observed them after T_{12}. In the cells used during the present study spores are not visible before T_{24}. Differences between the strains of yeast employed in these studies and differences between cultural conditions, especially in the presporulation phase, are likely to account for the differences observed in the timing of sporulation events by different workers. For example both Croes (1967a) and Halvorson et al. (1969) used a highly aerated presporulation medium, whereas in the present study a much richer presporulation medium which was hardly aerated was employed.

The cytological changes that my micrographs record during the first period are relatively uninteresting in that they are limited to small increases in the numbers of membrane profiles visible in the cytoplasm. The method of fixing material, namely by potassium permanganate, for

examination in the electron microscope is thought to ensure optimal preservation of membrane structure, but precludes observation of structures such as the intranuclear spindle or nuclear spindle plaques. These structures are clearly visible in Moens' (1971) and Moens and Rapport's (1971a) micrographs of the early stages of sporulation. Unfortunately Moens and his colleague do not quote the time sequence of their published micrographs, so it is difficult to relate their time scale to that of cells used in this study.

The increase in dry weight of the developing ascus during this and later periods has been noted before (Croes, 1967a; Esposito et al., 1969), and has been attributed to the increased contents of protein, carbohydrate and lipid. However the ability of cells to retain water during this part of the sporulation process has not been reported previously. The reasons for this capacity to retain water are not quite clear. Although no data are available for changes in lipid composition, it is fairly evident from the changes apparent at T_{24} that most of the lipid fractions increase in size from T_0 to T_{12}. Such an assumption is supported by the incorporation of acetate into lipids, recorded during this period. An increase in the amount of lipid from T_0 to T_{12} would be required for the synthesis of new membranes. Against this background of increase in the lipid content of developing asci it is perhaps surprising that cells are able to retain water in such an increasingly hydrophobic environment. It must be remembered that other compounds which exhibit structural affinities for water, notably proteins and polysaccharides, also increase in amounts during this period. Structural water associated with protein or polysaccharide may well explain the increased water-retaining capacity. Just such a compound could conceivably be produced in response to sporogenesis and thus might be absent from vegetative

cells. In this case it would represent a spore-specific component.

Period 2: T_{12} to T_{18} The sudden appearance in the cytoplasm of numerous lipid vesicles is characteristic of this phase. The number of membrane profiles visible in the cytoplasm, which increased during the previous period, continues to increase during this period. The vesicles appear as randomly-situated electron-transparent areas, which are approximately 0.3um in diameter.

It is known that vesicles, seen as stained granules by optical microscopy (Pontefract and Miller, 1962), as empty vesicles in thin sections by electron microscopy (Hashimoto et al., 1960; Lynn and Magee, 1970), or as membrane-bound spherical granules with a structured content in freeze-etched specimens (Guth et al., 1972), increase in number during ascosporogenesis. It is also generally thought that such vesicles are composed mainly, if not exclusively, of lipid (Matile et al., 1969).

For the same reasons as discussed in the previous section of this discussion it is likely that the content of lipid in developing asci increases in the T_{12} to T_{18} period. Clearly some of this newly synthetised lipid will become sequestered in the lipid vesicles. In this context the increases in the sizes of the triacylglycerol fraction and sterol-ester fraction during the first 24h of ascus formation are particularily impressive. The cellular location and physiological significance of these lipids in yeast is as yet incompletely understood. Hunter and Rose (1972) and Hossack, Wheeler and Rose (1973) have shown that sterol esters and triacylglycerols are mainly

located in low-density structures, probably vesicles perhaps similar to the low-density lipoproteins of mammalian serum (Margolis. 1969). Serum lipoproteins are utilised for transporting lipids, mainly triacylglycerols and cholesterol in the blood stream. Robinson (1964) has suggested that triacylglycerols in lipoproteins are specifically involved in mammalian fatty-acid transport. Triacylglycerols are commonly regarded as a reserve energy storage form (Robinson and Mead, 1970) and.tend to accumulate in yeast grown at low temperature (Hunter and Rose,. 1972), possibly as a response to ensure survival under extreme conditions. It is interesting to note that triacylglycerols accumulate in sporulating yeast, under conditions of comparative starvation. In the present study the sporulation culture was incubated 5^{o}C lower than the presporulation culture, which may explain the increased synthesis of triacylglycerol as a temperature effect. However the increase in triacylglycerol noted by Hunter and Rose (1972) was far smaller as a result of a difference of 15^{o}C in incubation temperature than the triacylglycerol increase presented here as a result of sporulation. Also worthy of note is the fact that other organisms, notably Dictyostelium, accumulate compounds which are generally held to be storage components during the course of differentiation which is also initiated during starvation conditions (Garrod and Ashworth, 1973).

Margolis (1969) has reviewed published data on the structure of low-density lipoproteins and concluded that triacylglycerol and cholesterol ester are in a central core partially covered by protein. This protein may be associated in a membrane-like structure as it has been suggested that the phospholipid and free cholesterol of this lipoprotein are near the surface.

It seems likely that the increased contents of triacylglycerol and

sterol ester might be associated with the formation of lipid vesicles during sporulation. However the contents of these two classes of lipid increase steadily from T_{24} to T_{120}, whereas the formation of lipid vesicles, as seen in electron micrographs, ceases after T_{24}. Indeed it appears that the number of vesicles declines once ascospores are visible. Thus the appearance of vesicles cannot be too closely correlated with synthesis of sterol ester and triacylglycerols. If the vesicles do contain these classes of lipid, some lipid must also be located in other ascan structures during later stages of development. However the asynchrony of sporulation in yeast is such that the number of asci formed in a sporulating culture continues to increase from T_{24} to T_{120} and, consequently, it is difficult to define accurately the period of appearance of lipid vesicles and relate this to changes in lipid composition.

Period 3: T_{18} to T_{30} This stage is characterised by the division of the nucleus and the delineation of the individual ascospores. The nuclear membrane remains intact during meiosis and until the spore nuclei are pinched off by the spore delimiting membranes. These findings are in good agreement with the observations by Moens (1971) and Moens and Rapport (1971a) of uninuclear meiosis, in which meiosis I and meiosis II are thought to take place within the confines of a single nuclear mass. Guth *et al.* (1972) confirmed these observations using freeze-etched samples of *Sacch. cerevisiae*.

The spore-delimiting membranes are described in this thesis as two parallel unit membranes. The inner of the two membranes eventually becomes the spore plasma membrane and the outer membrane becomes the

spore investing membrane.

The electrokinetic properties of cells are due to iogenic groups present on the surface of the cell (Richmond and Fisher, 1973). Lipid material, other than phospholipid, on the surface is not revealed by simple pH-mobility studies (Hill, James and Maxted, 1963). However the action of surface-active agents (for example, sodium dodecyl sulphate) will produce changes in mobility which indicate the presence of surface lipid material (Dyar and Ordal, 1946). The electrophoretic-mobility pattern of mature ascospores of Sacch. cerevisiae suggests the presence of a surface protein layer. The electrophoretic-mobility pattern of ascospores in the presence of sodium dodecyl sulphate indicate that no lipid material is associated with this protein.

The Danielli-Davson model (1934) as modified by Robertson (1958) is widely accepted as representing the basic ultrastructure of biological membranes. In this model the phospholipids are arranged as a bimolecular leaflet in a continuous sheet, with a layer of protein overlying each side. Electrokinetic studies of particles completely invested by such a unit membrane would only detect the protein part of that membrane. The electron micrographs presented in this thesis show that developing ascospores possess an investing membrane. No micrographs of mature ascospores are available, but it is likely that this membrane is retained during maturation of the spores. This hypothesis is supported by the electrophoretic evidence for a surface protein layer on yeast ascospores.

Further evidence is available to support this hypothesis. The surface of yeast ascospores is known to be highly hydrophobic (Emeis, 1958; see also Results section). The proteins which can be separated from

biological membranes are characteristic for a high content in 'hydrophobic' amino acids, for example glycine, alanine, phenyl alanine, leucine and isoleucine (Tria and Barnabei, 1969). The structural protein found in mitochondrial membranes is especially hydrophobic (Criddle and Willemot, 1969).

The spore-delimiting membranes are equivalent to the 'prospore' wall described by Moens (1971) and the 'forespore membranes' described by Guth et al. (1972). Each of these structures is bilaminar and demarcates a spore by progressive extension around a lobe of the post-meiotic nucleus. It has been shown here that the spore wall develops by insertion of spore-wall material between the spore-delimiting membranes, which move apart to accomodate this development. This method of spore-wall formation has often been described in species of higher ascomycetes, but has only twice been described for Sacch. cerevisiae (Marquardt, 1964; Lynn and Magee, 1970). These workers showed that the spore wall develops between the spore plasma membrane and a second membrane which is concentric with it.

Delineation of ascospores and spore wall formation in ascomycetes has been fully described in the Introduction to this thesis. In certain ascomycetes, spore-delimiting membranes (which fuse to form an 'ascus vesicle' (Carroll, 1967) in Saccobolus and Ascodesmis (Carroll, 1967), Pyronema (Reeves, 1967), Xylosphaera (Beckett and Crawford, 1970), Ascobolus (Oso, 1970) and Hypoxylon (Greenhalgh and Griffiths, 1970)) arise from membrane-bound vesicles which originate from the nuclear envelope and endoplasmic reticulum (Carroll, 1967; Beckett and Crawford, 1970). However Guth et al. (1972) point out that the origin of the spore-delimiting membranes is obscure in Sacch. cerevisiae, an observation which leads them to state: "the process of formation of

ascospore wall (in Sacch. cerevisiae) is unlike that reported for other ascomycetes...". I believe this conclusion to be mistaken since it is clear that, firstly, the spore-delimiting membranes in Sacch. cerevisiae and the ascus vesicles of higher ascomycetes are essentially similar both in structure and function; and secondly, the process of spore-wall development between two opposing membranes is very similar in both instances.

Most previous reports of changes in lipid content during sporulation in Sacch. cerevisiae have been based on cytological observations either with the light microscope (Pontefract and Miller, 1962) or with the electron microscope (Hashimoto et al., 1958; Lynn and Magee, 1970). Chassang et al. (1972) reported on the basis of gravimetric assays of extracted lipids, that the content of lipid in cells of Sacch. cerevisiae doubled during sporulation. This corresponds poorly with the fourfold increase in total lipid, observed in the present study, between T_o and T_{24}. Also the total amount of lipid extracted from cells (1.6mg/10^9 cells) and asci (3.2mg/10^9 asci) by Chassang and his colleagues is much lower than my corresponding figures (2.15mg/10^9 cells and 8.40mg/10^9 asci, respectively). These variances may be explained by strain differences, the use of different culture media, and variation in techniques for extraction and estimation of lipid. These workers employed a sporulation medium which contained phosphate; the presence of phosphate in the medium is known to affect the lipid composition of yeast (Johnson, Brown and Minnikin, 1973). Extraction of lipids was performed by refluxing cells with an azeotropic mixture of benzene, ethanol and water. This extract was divided into a saponifiable and an unsaponifiable fraction, and the two fractions were estimated gravimetrically.

Formation of four ascospores within an ascus involves a considerable increase in the amounts of new plasma membrane and, as shown by the micrographs, it involves the formation of spore-investing membrane and large amounts of endoplasmic reticulum membranes. A rough calculation indicates that formation of four ascospores in asci of Sacch. cerevisiae, using average dimensions from the electron micrographs, involves the synthesis of approximately 100% new membrane as a result of formation of spore plasma membrane and spore-investing membrane for each ascospore. The doubling in content of free sterol from T_0 to T_{24} is more than accounted for by the formation of new membrane and the subsequent tenfold increase from T_0 to T_{120} more than covers the calculation for new membrane. The threefold increase in phospholipid from T_0 to T_{24} more or less accounts for the synthesis of new membrane.

As mentioned earlier it is only possible to speculate on the physiological role of the increased contents of sterol ester and triacylglycerol, and the physiological role of vesicles which may contain these lipids. In this respect the location of the vesicles within the cytoplasm as sporulation proceeds may offer some clues. Pontefract and Miller (1962), in an optical microscope study, were the first to note the association between fat globules and the developing spore wall. They observed 'masses of fat' approximately at the location of the future spore wall and observed fat to be in contact with both the inside and the outside surfaces of the maturing spore wall. When the spores had matured the amount of fat present in the asci was visibly lowered. Lynn and Magee (1970) noted the association between lipid vesicles and the immature spore plasma membrane and later on between lipid vesicles and the developing spore wall. A similar association between osmiophilic (lipid) vesicles and the 'prospore wall' was demonstrated by Moens (1971) and between lipid granules and the

'forespore membranes' in freeze-etched material was demonstrated by Guth et al. (1972). In the present study lipid vesicles were observed to be randomly situated within the cytoplasm of cells during the previous period (T_{12} to T_{18}). However in the present period (T_{18} to T_{30}) the lipid vesicles have become translocated and arranged around the outside of the spore-delimiting membranes. This displacement of vesicles from one site in the cell to a more closely defined site is an example of spatial development which I spoke of earlier. What is the nature of the propellant which moves the lipid vesicles from one site in the cytoplasm to another? It must be remembered that the electron micrographs present a false picture of the solidity of the internal organisation of cells. A yeast cell, when observed as a living, wet preparation under the phase-contrast microscope, is seen to be full of small refractile granules. Brownian motion rapidly propels these granules to all parts of the cell.

Consequently when spore-delimiting membranes are being laid down lipid vesicles will constantly be colliding with them. At some point in time these membranes and the lipid vesicles are specifically attracted to one another and lipid vesicles remain arranged around them. The nature of this specificity is as yet unknown.

It seems likely that these lipid vesicles are in some way involved in the formation of the spore wall. It is difficult to specify the exact nature of this involvement, but the vesicles may supply previously synthetised wall material, or they may act as repositories for wall synthetising enzymes. The hypothesis that lipid vesicles are involved in spore-wall formation is supported by several other observations. Firstly, the presence of lipid vesicles within the spore-investing membrane during the time when the spore wall is being laid down. As

the spore wall matures the blebs empty and collapse. Secondly, there is a very rapid incorporation of acetate into sterol esters and triacylglycerols during the period T_{24} to T_{30} which is the period during which spore walls are being laid down. The burst of activity in lipid metabolism during this period has also been noted by Esposito et al. (1969) and Miyake et al. (1971). Thirdly, vesicles of the type found in the present study have been implicated in the secretion of invertase into the periplasm of yeast cells (Holley and Kidby, 1973) and in extrusion of cell wall material into the bud wall and septum of dividing yeast (Sentandreu and Northcote, 1969) and into growing bud cell-wall of heterobasidiomycetous yeast (McCully and Bracker, 1972).

Bartnicki-Garcia (1973) has proposed a unitary model of cell wall growth at the apices of fungal hyphae. In this model cytoplasmic vesicles secrete lytic enzymes into the apical wall, which as a result is weakened and stretches under the influence of high turgor pressure. Wall synthetising enzymes are then secreted into the stretched wall by other vesicles. Vesicles containing amorphous wall material; in a largely or entirely preformed state, deposit their contents against the wall, and are possibly transported across the membrane via lipid intermediates.

Although the data presented in this thesis show an increase in the fatty-acid unsaturation in lipids as sporulation progresses, there are no data to suggest a preferential location, if any, of unsaturated fatty acids in ascan lipids. An increase in the degree of unsaturation of fatty-acid residues when the growth temperature of microorganisms is lowered is well known (Hunter and Rose, 1972). It is possible that the extra mobility which unsaturated fatty acids confer on sterol esters

and triacylglycerols (Farrell and Rose, 1967) might be important in vesicles which transport wall materials or enzymes.

Anaerobically-grown cells of yeast are auxotrophic for an unsaturated fatty acid (Andreason and Stier, 1954). The factor controlling this effect is the requirement for molecular oxygen by the fatty-acid desaturase enzyme. Since induction of sporulation involves a change from a semi-anaerobic culture to a vigourously aerobic culture, the increased availability of oxygen in the sporulation medium may control the increased levels of unsaturated fatty-acid residues. It is known that an increase in the dissolved-oxygen tension during growth of either nitrogen-limited or glucose-limited chemostat cells of Candida utilis leads to an augmented synthesis of unsaturated fatty-acid residues (Brown and Rose, 1969).

The presence of unsaturated fatty acids in membrane phospholipids prevents them packing as closely together as phopholipids containing saturated fatty acids (van Deenen, 1965). Thus increased synthesis of unsaturated fatty acids would lead to synthesis of more expanded membranes. De Gier, Mandersloot and van Deenen (1968) studied the permeability of liposomes (layered latticed liquid crystals) and showed that the introduction of lipids with unsaturated fatty-acid residues into these artificial systems enhanced their permeability to non-electrolytes. Incorporation of cholesterol into liposomes had the reverse effect and decreased permeability. Similar effects of permeability and leakage have been reported by de Gier, Haest, Mandersloot and van Deenen (1970). It is possible that the greater degree of unsaturation of fatty acids in yeast lipids during sporulation and the changes in membranes which stem from this are not requisite to sporulation but merely an interesting but nevertheless unimportant by-product of sporulation.

It has been known for a long time that acetate is an excellent substrate for ascosporogenesis in Sacch. cerevisiae (Fowell, 1969). The reasons for this are not fully understood. Croes (1967b) has suggested that the triggering action of acetate is a speeding up of energy supply processes immediately after yeast is transferred to acetate-containing media. This results in an insufficiency of the glyoxylate cycle. The glyoxylate cycle is required to provide carbon fragments for synthesis of amino acids. Acetate also plays a very important role in lipid biosynthesis. Fatty acids are formed by repeated addition of malonyl-CoA (itself formed from acetyl-CoA) onto a primer acetyl-CoA molecule. Sterols are formed by repeated condensations between acetyl-CoA molecules to give an isoprenoid unit, which subsequently undergoes successive condensations to give the C_{30} hydrocarbon (squalene). Cyclisation of squalene eventually leads to sterol formation. The rapid biosynthesis of lipids during sporulation may be facilitated by the use of acetate as a substrate. It is possible that the efficiency of acetate as a presporulation substrate is due in part to its central position in lipid metabolism, which as we have seen, plays an important role in sporogenesis.

Period 4: T_{30} to T_{120} This period is characteristically one of consolidation. The biosynthetic activity of asci in this phase is greatly diminished, although some lipid fractions do continue to increase upto T_{120}. Although electron micrographs are not available for this period it is unlikely that any major changes occur in fine structure, with the exception that spore walls continue to thicken until maturity is reached.

INHIBITION OF SPORULATION BY AMMONIUM IONS

In the past, various reports (Miller and Hoffmann-Ostenhof, 1964; Fowell, 1967) have indicated that the presence of nitrogenous compounds, especially ammonium salts delay or completely inhibit sporulation in yeast. It has been generally assumed (Tingle, Klar, Henry and Halvorson, 1973) that this inhibition is effective during the whole sporulation process. However it has been demonstrated by the present study that ammonium ions are only really effective in inhibiting sporogenesis if present from T_{20} to T_{30}. If ammonium ions are present before or after this period, but not present during this period, then sporulation is not inhibited.

The mechanism by which ammonium ions inhibit sporulation is not known. It may be related to its ability to delay synthesis of some enzymes whilst being able to stimulate synthesis of others. For example, the addition of ammonium ions to starved yeast stimulates induction of α-glucosidase (Halvorson and Spiegleman, 1953) and stimulates the activity of phosphofructokinase (Atzpodien and Bode, 1970). A consequence of stimulation of both of these enzymes would be the enhancement of glycolysis. Alternatively incorporation of ammonium into glutamate, which is catalysed by NADP-linked glutamate dehydrogenase, may stimulate the direct oxidative pathway of yeast (Holzer and Witt, 1960). Glycolysis is known to repress the tri-carboxylic acid cycle (Ephrussi, Slonimski, Yotsuyanagi and Tavlitzki, 1956) and intermediaries of both these pathways (glycolysis and the direct oxidative pathway) have been proposed as co-repressors of iso-citrate lyase (Kornberg, 1966; Guerritor, Hanozet and Coccucci, 1969). Gosling and Duggan (1971) found that adaptation to acetate oxidation by bakers yeast was considerably delayed in the presence of ammonium

ions. This delay was found to be due to a delay in the increase in activity of isocitrate lyase normally associated with acetate adaptation.

Croes (1967b) has suggested that sporulation in the presence of acetate requires both the tricarboxylic acid cycle and the associated glyoxylate by-pass to be fully operational. Miyake et al. (1971) have shown that the glyoxylate cycle predominates during sporulation and that there is a marked increase in the activity of isocitrate lyase during the first few hours of sporulation. Isocitrate lyase is responsible for converting isocitrate to glyoxylate and succinate, which is the first step of the glyoxylate pathway.

It would appear therefore that ammonium effects changes in metabolism, primarily by inhibiting isocitrate lyase and thus inhibiting acetate adaptation, which should inhibit or delay the onset of sporulation. However results presented in this thesis indicate that ammonium ions do not inhibit the onset of sporulation. It is possible that sporulation is delayed for some time if ammonium is present during the period of adaptation to acetate. The experiments used in the present study would not have detected if any such delay occurred. When ammonium ions were present between T_0 and T_{20} and then removed, the cells were allowed to incubate for a further 100h which would allow sufficient time to elapse and the delay overcome. On the basis of the argument presented above, once adaptation to acetate had been accomplished then sporulation should be relatively insensitive to inhibition by ammonium ions.

It appears that this hypothesis does not apply. Sporogenesis is most sensitive to ammonium ions when they are present between T_{20} and T_{30},

long after acetate adaptation has been completed. The present study has shown that the most important processes occurring during this phase are delimitation of individual ascospores and development of the spore wall. It is quite conceivable that ammonium ions may irreversibly affect induction of enzymes or synthesis of enzymes specific to these two processes, in a way analagous to repression of enzymes involved in acetate adaptation.

In conclusion ascosporogenesis in Saccharomyces cerevisiae is a complex process involving many different sequences of events. In the work described in this thesis I have attempted to collate only two of these sequences, namely lipid composition and fine structure. I have also investigated the effect of ammonium ions on ascosporogenesis. In this way, by dissecting out of the overall process two or three discrete sequences and proceeding to relate one with another, it is possible to gain further knowledge and insight of this very complex process of sporogenesis.

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APPENDIX

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Evidence for a Surface Protein Layer on the *Saccharomyces cerevisiae* Ascospore

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Electrophoretic measurements on *Saccharomyces cerevisiae* ascospores indicated the presence of a surface protein layer which can be removed by papain, chymotrypsin or 8 M urea.

Little is known about the composition of the outer layers of the yeast ascospore. Because of the hydrophobic nature of yeast ascospores and their affinity for Sudan Black, several workers (3, 9, 13) suggested that the outermost layer is composed of lipid. This conclusion is not consistent with the presence, on the outside of all yeast ascospores so far examined (5, 8), of an electron-dense layer or with the marked ultraviolet-absorbing properties of this layer (12). This report deals with the electrophoretic properties of ascospores from *Saccharomyces cerevisiae* before and after treatment with various reagents. The data suggest that the yeast ascospore is covered with a layer of protein which overlays a thick spore wall probably composed of polysaccharide.

The strain of *S. cerevisiae* (DCL 740) was grown in the presporulation (nutrient broth plus 5% glucose and 1% yeast extract) and KCl (1.0%)-sodium acetate (0.5%) sporulation media recommended by Fowell (5). Approximately 60 to 65% of the cells sporulated after 5 days of incubation at 25 C in the sporulation medium. Asci and vegetative cells were harvested by centrifugation at 0 C and washed twice with water. A suspension of cells and asci (80 mg, dry weight, per ml of 50 mM sodium acetate buffer; *p*H 5.5) was supplemented with one-third volume diluted snail juice (7) and incubated at 30 C for 24 hr. The cells and asci were harvested, and the ascospores were released from asci by subjecting a cold-water suspension to sonic treatment for 3 min with an ultrasonic disintegrator (Measuring & Scientific Equipment, Ltd.) at 20 kc per sec. Release of ascospores from asci was monitored by microscopic examination.

Figure 1 shows the *p*H-mobility curve for untreated ascospores. The shape of the curve is indicative of an amino-carboxyl surface, probably of protein (14). Further evidence against the presence of a lipid surface came from the finding that the mobility of spores was not affected by incorporating sodium dodecyl sulfate (0.1 to 0.001 mM) in 0.01 M phosphate buffer (*p*H 7.0) (4). Digestion of spores with trypsin did not alter the shape of the *p*H-mobility curve, although it increased the mobility values at high and low *p*H values. Digestion with pepsin or chymotrypsin changed the electrophoretic mobility pattern to one characteristic of a negatively charged surface (Fig. 2). A similar effect was produced after treatment of isolated spores with 8 M urea (Fig. 2). Electron micrographs of thin sections through ascospores showed that 8 M urea completely removed the electron-dense layer surrounding the spores. Ascospores which had been treated with papain or chymotrypsin retained small amounts of electron-dense material on the surface.

These data suggest that the outside of the ascospores from *S. cerevisiae* is coated with protein, a conclusion which is in agreement with the electron-dense (5.8) and ultraviolet-absorbing (12) properties of this layer. However, the data do not preclude the possibility that the outer layer is composed of a lipoprotein, the lipid moiety of which lies below the surface of the spore. The hydrophobic character of the yeast ascospore suggests that the surface protein may resemble the structural protein found in mitochondrial membranes (1). Marquardt (10) reported that the outside layer of the yeast ascospore is synthesized by the ascal protoplasm rather than by the spore. The electrophoretic mobility of ascospores after digestion with pepsin or chymotrypsin, or treatment with 8 M urea, suggests that the underlying electron-transparent spore wall is probably composed of polysaccharide which is not covalently linked to the surface protein. Eddy and Rudin (2) showed that

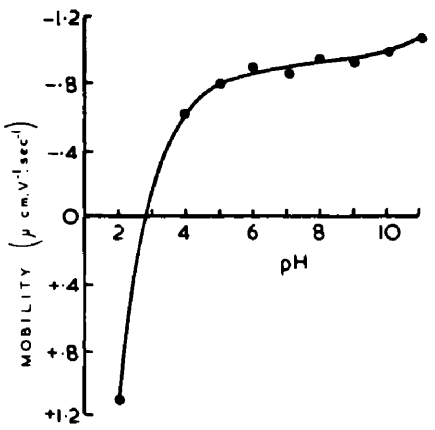

FIG. 1. *Effect of pH value on electrophoretic mobility of ascospores from Saccharomyces cerevisiae DCL 740. Electrophoretic mobilities were measured by a modification of the technique of Somers and Fisher (14). Movement was timed over 180 μm in both directions (current reversal), and each mobility value was obtained from at least 20 observations. The standard error of the mean was less than 4%. Electrophoretic measurements were made by using suspensions containing about 10^6 ascospores/ml. Spores were washed twice in the appropriate buffer before suspension in the HCl-NaCl or barbiturate-acetate buffer of the required pH value (6). All the buffers had an ionic strength of 0.05.*

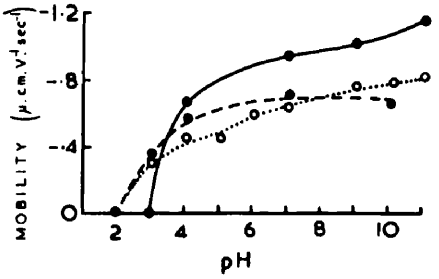

FIG. 2. *Effect of digestion with pepsin* (○——○) *and chymotrypsin* (●——●) *and treatment with 8 M urea* (●···●) *on the electrophoretic mobility of ascospores from Saccharomyces cerevisiae DCL 740. Mobility measurements were made as described in the legend for Fig. 1. Enzyme digestion was done by incubating suspensions of washed ascospores (10^6/ml) containing 100 μg of enzyme/ml for 6 hr at 35 C. The spores were washed three times with water before mobility measurements were made. The pH value of the suspension containing pepsin was 2.5 and 7.5 in the suspension containing chymotrypsin.*

all of the yeasts they examined had walls with a net negative charge which may be attributed to the phosphodiester linkages between mannose residues in the cell-surface mannan (11). However, stationary-phase cells and asci of *S. cerevisiae* DCL 740 have no net charge which suggests that their surface layers differ in composition from the ascospore wall. The known variability of mannose:phosphate ratio in the mannans of different strains and species of yeast could well account for the lack of charge in DCL 740.

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Changes in the Lipid Composition and Fine Structure of *Saccharomyces cerevisiae* During Ascus Formation

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Eighty to ninety percent of vegetative cells of *Saccharomyces cerevisiae* DCL 740 incubated in KCl-acetate medium form asci, the majority of which are four-spored. Ascospores are visible in asci after about 24 hr, and spore formation is complete after about 48 hr. The dry weight of the cells increases by about 75% during 48 hr of incubation, while the lipid content of the cells increases by a factor of four. The increase in lipid content is attributed mainly to an increased synthesis of sterol esters and triacylglycerols and to a lesser extent of phospholipids. The phospholipid and sterol compositions do not change appreciably, but there is a marked increase in the proportion of unsaturated fatty acid residues in ascan lipids. Uniformly labeled ^{14}C-acetate is incorporated mainly into sterol esters and triacylglycerols and phospholipids. Pulse-labeling by adding acetate-*U*-^{14}*C* to sporulating cultures and harvesting after a further 6 hr of incubation reveal two main periods of acetate incorporation, namely between 0 and 18 hr, and between 24 and 30 hr. Electron micrographs of thin sections through developing asci show that the principal changes in fine structure occur between 18 and 24 hr and include the appearance of numerous electron-transparent vesicles which become aligned around the meiotic nucleus, and the laying down of extensive endoplasmic reticulum membranes. Changes in fine structure are discussed in relation to the alterations in lipid content and composition of asci.

Many of the changes in composition that occur when diploid cells of *Saccharomyces cerevisiae* form asci containing one to four haploid ascospores have been described (see Fowell [14] for a review). These include an increase in cell volume and dry weight (8) which is accompanied by an increase in the contents of deoxyribonucleic acid (DNA) and carbohydrate (8). The contents of total ribonucleic acid (RNA) and protein (8) also change, although in a variable fashion. Moreover, it has been reported that yeast asci are richer in lipid than vegetative cells (17, 18, 32), and fat globules (23) are claimed to have been detected in electron micrographs of thin sections through asci. On the whole, however, the changes in lipid content and composition that accompany ascus formation in *S. cerevisiae* are poorly described especially in view of the obviously extensive synthesis of membranes that takes place. The present paper describes changes in the lipid composition during ascus formation in a strain of *S. cerevisiae*, and also the extent to which acetate, which is the preferred carbon source during ascus formation, is incorporated into cell lipids. Changes in lipid composition are related to morphological events that occur during ascus development as revealed by electron microscopy.

MATERIALS AND METHODS

Organism. The yeast used in this study was a strain of *S. cerevisiae* DCL 740 kindly provided by R. R. Fowell. It was maintained on slopes of malt wort-agar as described by Dixon and Rose (10). Stock cultures were stored at 4 C. This strain of yeast produces a high proportion (80-90%) of four-spored asci. When the sporulating ability of the yeast declined, single-cell isolates were obtained by plating on malt wort-agar medium. The majority of these isolates produced a high proportion of asci.

Growth of cells. Cells were grown in a presporulation medium containing 1.3% nutrient broth (Ox-

oid), 5% glucose, and 1% yeast extract (Oxoid); *p*H 6.3 (14). Portions (1 liter) of medium were dispensed into 21 round, flat-bottomed flasks which were plugged with cotton and sterilized at 115 C for 15 min. Batches of medium were inoculated with a loopful of cells from a slope culture and were incubated at 30 C as described by Patching and Rose (30), except that the magnetic stirrer was rotated only at about 200 to 300 rotations per min. After 40 hr, the culture was in the stationary phase of growth (about 4.75 mg dry weight or 1.50×10^8 cells/ml). The cells were then harvested by centrifugation at 12,000 × *g* at 3 C, washed twice with water, and resuspended in water.

Production of asci. Sporulation of cells was induced by incubating vegetative cells in a sporulation medium containing 0.5% (w/v) sodium acetate and 1.0% (w/v) KCl (*p*H 7.0; 14). Batches of sporulation medium (1 liter) were dispensed into 2-liter round, flat-bottomed flasks and inoculated by adding an aqueous suspension of washed vegetative cells to a density of 0.44 mg dry weight (1.44×10^7 cells) per ml. Suspensions were incubated at 25 C with rapid stirring (30). Production of asci was followed by removing portions of suspension and counting the numbers of two-, three-, and four-spored asci by using a hemocytometer slide. A cell with an attached bud was counted as one cell. At least 300 cells or asci were counted. Ascospores were visible in asci after about 24 hr of incubation, and spore formation was complete after about 48 hr. Nevertheless, the suspensions were incubated for up to 120 hr to allow the spores to ripen. Asci and cells were harvested from the suspension by centrifugation at 12,000 × *g* at 3 C. They were washed twice with water, freeze-dried, and stored at −20 C in the presence of a desiccant.

Dry weight measurements. Dry weight measurements were made on cells and asci that had been freeze-dried or dried to constant weight at 80 C under reduced pressure. These methods gave virtually identical values for vegetative cells, but not with developing asci (see Table 1).

Extraction of lipids. Lipids were extracted from vegetative cells and asci by a modification of the procedure used by Letters (21). Portions (500–800 mg) of freeze-dried material were extracted with 15 ml of ethanol at 80 C for 15 min. The residue was extracted at room temperature (18–22 C) with 3 × 30 ml of chloroform-methanol (1:1, v/v) and made up to 2 ml. The total lipid content of portions of this extract were then determined as described by Hunter and Rose (20).

Analysis of phospholipids. Total phospholipid content of the lipid extract was determined by assaying the phosphorus content of a 25-μliter portion of the extract by the method of Bartlett (1) or Chen et al. (7). Values for the phosphorus content were converted to phospholipid by multiplying by 25 (which assumes an average molecular weight of 800 for a phospholipid). Individual phospholipids in the extract were separated by two-dimensional thin-layer chromatography using plates (20 by 20 cm) coated with a layer (0.25 mm) of Kieselgel PF_{254} (Merck). Plates were developed in the first direction with chloroform-methanol-ammonia (0.88 specific gravity) (65:35:5, v/v/v), and in the second direction with chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5, v/v/v/v/v). Individual phospholipids were identified by using standard preparations and specific spray reagents (9).

Analysis of neutral lipids. Neutral lipids in extracts were separated quantitatively by two-dimensional thin-layer chromatography. Plates (20 by 20 cm), with a layer (0.75 mm) of Kieselgel $PF_{366 + 254}$ (Merck) were washed overnight in chloroform. Plates were developed with diisopropyl ether-acetic acid (96:4, v/v) to a distance 9 cm above the origin and then in the same direction with petroleum spirit (40–60 C)-diethyl ether-acetic acid (90:10:1, v/v/v) to a distance 16 cm above the origin. Bands of lipid were located with an ultraviolet lamp and identified with simultaneously run standards. Phospholipids located at the origin were eluted from the silica gel with 2 × 3 ml of chloroform-methanol-water (5:5:1, v/v/v) followed by 3 ml of methanol and 3 ml of methanol-acetic acid-water (95:1:5, v/v/v). The phospholipid content of the extract was assayed as already described. Sterols and sterol esters were eluted from the silica gel with 3 × 3-ml portions of chloroform-methanol (4:1, v/v) and assayed by a modification of the Liebermann-Burchard reaction described by Moore and Baumann (28). Gas-liquid chromatography revealed that the extracts contained only very small amounts of sterols that lack the 5,7-diene grouping. Consequently, only 5,7-diene sterols, which are the fast-reacting sterols in the Liebermann-Burchard assay, were determined. Sterol contents were calculated from a standard curve prepared with ergosterol. Sterol esters were dissolved in 0.1 ml of benzene and saponified by refluxing for 2 hr with 0.4 ml of 10% (w/v) KOH in 90% (v/v) ethanol in a tube fitted with a cold-finger condenser. The sterols liberated were assayed as already described. Diacylglycerols and triacylglycerols were eluted from the silica gel by 2 × 3-ml portions of chloroform followed by 2 × 3-ml portions of diethyl ether. The first ether extraction was performed in a cold Rotary Evapomix (Buchler Instruments Inc.) without a vacuum for 10 min. Diacylgycerols and triacylglycerols were assayed by a chromotropic acid method (39). Contents are expressed as dipalmitin- and tripalmitin-equivalent, respectively, derived from a standard curve prepared with tripalmitin. Free fatty acids were eluted from the silica gel by 2 × 3-ml portions of diethyl ether-methanol (9:1, v/v) followed by 2 × 3-ml portions of chloroform. They were assayed by the method of Heinen and de Vries (19), and contents were related to oleic acid-equivalent by using a standard curve. Squalene was not eluted from the silica gel before being assayed by the method of Trappe (38), which involves bromination of squalene and iodometric titration of the unreacted bromine.

Sterols were extracted from freeze-dried cells by hydrolysis followed by saponification. Yeast (100 mg) was refluxed with 30 ml of 0.33 N HCl for 1 hr. The supernatant liquid was extracted with 3 × 30 ml of diethyl ether, and the residue was refluxed with 5 ml of 7 N KOH for 2 hr at 130 C. The reaction mixture was extracted with 2 × 25 ml of cyclohexane with

shaking. The diethyl ether and cyclohexane extracts were pooled and analyzed for total sterol by the Liebermann-Burchard method (38) and for ergosterol by the ultraviolet extinction method of Shaw and Jefferies (36).

Gas-liquid chromatography. Samples were analyzed using a Pye series 104 model 64 chromatograph, with flame ionization detectors. Sterols were prepared for gas-liquid chromatography by dissolving the lipid extract in 1 ml of benzene and refluxing with 4 ml of 1.78 M KOH in 90% (v/v) ethanol for 2 hr. Water (10 ml) was added, and the *p*H value was adjusted to 1.0 with HCl. The mixture was then extracted with 3 × 10 ml of diethyl ether, and the extracts were dried over sodium sulfate before being concentrated in vacuo. Free sterols were purified by thin-layer chromatography with petroleum spirit-diethyl ether-acetic acid (70:30:2, v/v/v) and eluted from the silica gel with 150 ml of diethyl ether. Trimethylsilyl ethers were prepared by dissolving the sterols in 1 ml of dry pyridine, adding 0.2 ml of hexamethyl disilazane and 0.1 ml of trimethyl chlorosilane (37). The mixture was shaken and left at room temperature for 15 min. Excess reagents were evaporated off under a stream of nitrogen gas, and the products were taken up in chloroform. Alternatively, the reaction mixture was partitioned between petroleum spirit and water, and the petroleum spirit extract was concentrated and redissolved chloroform. Trimethylsilyl ethers of the sterols were separated on 3% OV 17 supported by 100–200 mesh Gas Chrom Q in a 9 ft by ¼ inch (ca. 2.7 m by 0.6 cm) stainless-steel column. The column temperature was 225 C with a nitrogen gas flow rate of 70 ml/min; the detector oven temperature was 300 C.

Methyl esters of fatty acids were prepared by refluxing 10 to 20 mg of extract in 0.5 ml of dry benzene with 4 ml of methanolic HCl (5%, w/v) for 2 hr. A tube of anhydrous $CaCl_2$ was fitted to the condenser. After adding 10 ml of water, the methyl esters were extracted with 3 × 10 ml of diethyl ether. These extracts were dried over $CaCl_2$ before being concentrated. The methyl esters were purified by thin-layer chromatography with petroleum spirit-diethyl ether-acetic acid (90:10:1, v/v/v) and were eluted from the silica gel with 150 ml of petroleum spirit-diethyl ether (1:1, v/v). The eluate was concentrated and redissolved in chloroform before the esters were separated on 15% polyethylene glycol succinate (PEGS) on 85-100 mesh Universal B, or 15% EGSS-X on 100-120 mesh Gas Chrom P. The PEGS was packed in a 5 ft by ¼ inch (ca. 1.5 m by 0.6 cm) glass column and maintained at 155 C; the detector oven was set at 200 C with a nitrogen gas flow of 60 ml/min. The EGSS-X was packed in a 5 ft by ¼ inch (ca. 1.5 m by 0.6 cm) stainless-steel column, maintained at 175 C with a nitrogen gas flow rate of 50 ml/min and the detector oven at 200 C.

Radioactive counting methods. ^{14}C activity in cells and extracts was measured by using a Beckman model CPM 200 liquid scintillation spectrometer (Beckman Instruments Limited, Glenrothes, Fife, Scotland). Yeast cells were removed from 5 ml of suspension containing acetate-*U*-^{14}C by filtering through Sartorius or Millipore membrane filters (1.2-μm pore size; 2.5-cm diameter) and washed with 3 × 10 ml of water, and the cells plus filters were dried to a constant weight in a vacuum desiccator. They were then placed in a scintillation vial containing 5 ml of scintillation liquid (toluene–2-methoxyethanol–2,5-diphenyloxazole; 3:2:0.003, v/v/w). Bands of silica gel containing various classes of lipid extracted from cells grown in the presence of acetate-*U*-^{14}C were scraped off thin-layer plates and transferred to scintillation vials containing 5 ml of scintillation liquid (toluene–2,5-diphenyloxazole; 1:0.003, v/w). All samples were counted up to 100 min or to a 2 σ statistical counting error usually equal to 1% or 2%. Readings were corrected for average background count by using blank areas of silica gel from thin-layer plates.

Electron microscopy. Vegetative cells and asci were fixed in 1% (w/v) aqueous potassium permanganate. Preliminary experiments showed that asci could be satisfactorily fixed while suspended in water, but with vegetative cells best results were obtained by first freeze-drying the cells and then resuspending them in water before chemical fixation. The optimal times for fixation were established with each developmental stage examined: vegetative cells, 4.0 hr; 6-hr asci, 5.5 hr; 12-hr asci, 5.0 hr; 18-hr asci, 4.5 hr; 24-hr asci, 4.0 hr. Fixed material was washed in water and suspended in liquid 1% (w/v) agar which, after setting, was cut into 1-mm^3 blocks. These blocks were dehydrated in a graded ethanol–water series, soaked in propylene oxide, and embedded in Epon. Blocks were stained for 1 hr during dehydration in 2% (w/v) uranyl acetate in 70% (v/v) ethanol. Sections were cut with a diamond knife on an LKB ultramicrotome, stained in lead citrate (33), and viewed with an AEI EM 6M electron microscope.

Chemicals. Standard lipids were supplied by Sigma Chemical Co., London, England; standard mixtures of fatty acid methyl esters and 15% EGSS-X on Gas Chrom P (manufactured by Applied Science Laboratories, Inc.) by Field Instruments Ltd., Richmond-upon-Thames, Surrey, England; and PEGS (15% on Universal B) and 3% OV-17 on Gas Chrom Q by Phase Separations Ltd., Queensferry, Flintshire, Wales. Silica gels manufactured by E. Merck A. G. were from Anderman and Co. Ltd., London, England. The sodium salt of acetic acid-*U*-^{14}C was obtained from The Radiochemical Centre, Amersham, Bucks., England. All other chemicals were analytical grade or of the highest purity available commercially. Chloroform and methanol were redistilled before use.

RESULTS

Total lipid contents of developing asci. Vegetative cells do not bud in sporulation medium, although as previously reported (8) they increase in dry weight and volume as ascospore formation proceeds. Initially, cells and developing asci were dried by freeze-drying, and it was shown that freeze-dried cells do not lose weight when dried at 80 C under

reduced pressure. However, as shown in Fig. 1, freeze-dried asci retained water, the amount of which increased as the asci developed. This increase in water retention occurred in two distinct periods, namely between 0 and 12 hr, and between 30 and 48 hr. The increase in dry weight is accompanied by an increase in the content of total lipid (Fig. 1), which is more rapid than the increase in dry weight. Values for the lipid content of vegetative cells showed good agreement between batches, but, as ascus formation proceeded, values for the lipid content varied somewhat between batches.

Changes in the lipid composition of developing asci. The increase in lipid content of cells during the first 24 hr of incubation in sporulation medium can be attributed mainly to an increased production of sterol esters, triacylglycerols, and phospholipids (Table 1). During this period, there were relatively small increases in the contents of free sterols, diacylglycerols, free fatty acids, and squalene. The phospholipid, diacylglycerol, and free fatty-acid contents of lipids from asci after 120 hr of incubation differed little from those in cells incubated for only 24 hr, but the contents of sterols, sterol esters, and particularly of triacylglycerols were much greater.

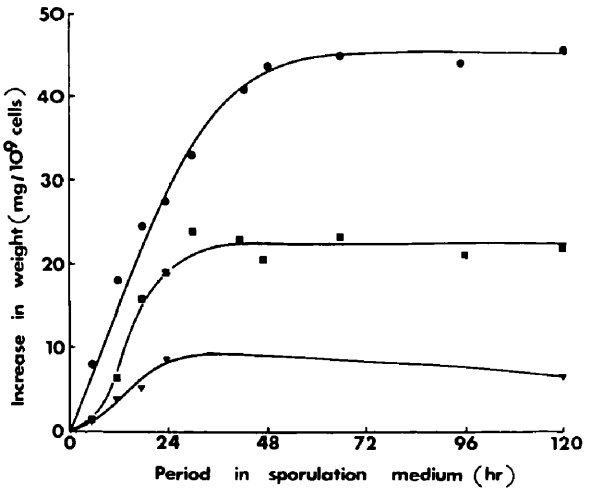


FIG. 1. *Increase in weight (freeze-dried cells, ●; heat-dried cells, ■) and content of total lipids (▼) of Saccharomyces cerevisiae DCL 740 during ascus formation. At zero time, the value for freeze-dried cells was 30.3 mg/10⁹ cells, and for heat-dried cells, 30.7 mg/10⁹ cells. The lipid content of cells at zero time was 2.31 mg/10⁹ cells.*

Vegetative cells contain large proportions of phosphatidylinositol, phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine, and smaller proportions of phosphatidic acid, bisphosphatidylglycerol, and *N,N*-dimethyl-phosphatidylethanolamine. The relative proportions of these phospholipids did not change appreciably during development of the asci up to 120 hr. There was a progressive increase in the proportion of unsaturated acids during ascus development, due mainly to an increased synthesis of $C_{16:1}$ and $C_{18:1}$ acids. Vegetative cells were grown in the presporulation medium at 30 C and incubated in the sporulation medium at 25 C. Growth temperature is known to influence the fatty acid composition of microorganisms (12), lower incubation temperatures favoring synthesis of unsaturated acids. Table 2 shows for comparison the fatty acid composition of cells grown in a presporulation medium at 25 C; when transferred to the sporulation medium, these cells gave rise to 5 to 10% fewer asci compared with cells that had been grown at 30 C. Nevertheless, the proportion of unsaturated fatty acids (68.1%) in vegetative cells grown in presporulation medium at 25 C is much lower than the value (77.9%), after 24 hr of incubation in sporulation medium, obtained for asci developing from cells grown in presporulation medium at 30 C.

Gas-liquid chromatography of sterols obtained from cells and asci either by saponification or by extraction with chloroform-methanol (Table 3) revealed two major components, with trace amounts of two others. One of the major components has a retention time (2.52 relative to cholestane) identical with that of ergosterol and was tentatively identified as such. The other major sterol, which is present in amounts equal to about half that of ergosterol, was at first tentatively identified by its relative retention time (3.00) as the tetraethenoid sterol

TABLE 1. *Changes in the lipid composition of Saccharomyces cerevisiae DCL 740 during ascus formation*[a]

Period in sporulation medium (hr)	Phospholipid	Sterol	Sterol ester	Diacyl-glycerol	Triacyl-glycerol	Free fatty acid	Squlene
0	0.80 ± 0.09	0.05 ± 0.01	0.08 ± 0.03	0.03 ± 0.01	0.14 ± 0.02	0.06 ± 0.02	0.01 ± 0.00
24	2.17 ± 0.13	0.09 ± 0.01	1.11 ± 0.28	0.21 ± 0.07	1.61 ± 0.23	0.08 ± 0.01	0.33 ± 0.04
120	2.24 ± 0.17	0.50 ± 0.09	2.92 ± 0.48	0.23 ± 0.10	5.24 ± 1.03	0.09 ± 0.04	0.25 ± 0.04

[a] Values quoted are milligrams per 10^9 cells with 95% confidence limits. Values for sterol ester were calculated assuming esterified sterol to be ergosterol palmitate.

TABLE 2. *Fatty acid composition of lipids of Saccharomyces cerevisiae DCL 740 during ascus formation*

Acid	Fatty acid composition[a]			
	0 hr	24 hr	120 hr	0[b] hr
12:0	3.47 ± 0.77	0.56 ± 0.19	tr[c]	0.77 ± 0.32
13:1	0.87 ± 0.02	tr	tr	tr
14:0	1.48 ± 0.29	1.41 ± 0.70	1.03 ± 0.02	1.29 ± 0.40
15:0	1.04 ± 0.08	0.60 ± 0.04	0.74 ± 0.09	tr
16:0	25.74 ± 2.98	15.57 ± 0.75	8.43 ± 0.73	23.03 ± 2.04
16:1	39.00 ± 0.93	44.24 ± 1.80	50.73 ± 1.68	31.40 ± 2.34
18:0	4.76 ± 1.02	4.95 ± 0.44	3.13 ± 0.86	6.80 ± 0.24
18:1	23.82 ± 2.16	33.32 ± 0.96	35.45 ± 0.58	36.71 ± 3.38
Percent saturated acids	35.45	23.09	13.70	31.89
Percent unsaturated acids	63.69	77.90	86.18	68.11

[a] Values quoted are the molar percentages of the total fatty acid (with 95% confidence limits) after 0, 24, or 120 hr in sporulation medium at 25 C.

[b] Cells grown in presporulation medium at 25 C instead of 30 C.

[c] tr indicates that the percentage of fatty acid was less than 0.50%. The following acids were also present at less than 0.5%: 7:0, 8:0, 9:0, 10:0, and 13:0.

TABLE 3. *Gas-liquid chromatographic determination of proportions of individual sterols (free and esterified) in developing asci of Saccharomyces cerevisiae DCL 740*

Retention time of sterol relative to cholestane[a] (hr)	Sterols extracted from cells by using:	Sterol content in cells incubated in sporulation medium[b]		
		0 hr	24 hr	120 hr
1.70	$CHCl_3$-MeOH	tr[c]	tr	tr
1.95	$CHCl_3$-MeOH	tr	tr	tr
2.52	$CHCl_3$-MeOH	62.0 ± 7.2	66.7 ± 3.0	60.6 ± 5.3
	Saponification	72.5 ± 6.5	63.9 ± 5.8	63.7 ± 8.4
3.00	$CHCl_3$-MeOH	35.2 ± 8.6	33.3 ± 3.0	39.4 ± 5.4
	Saponification	27.8 ± 6.6	34.4 ± 4.8	36.3 ± 8.4

[a] Sterols were separated by gas-liquid chromatography by using a column of OV-17.

[b] Values quoted are percent of the total sterol with 95% confidence limits.

[c] tr indicates trace (less than 0.50% of the total).

described by Longley, Rose, and Knights (22) and Hunter and Rose (20). However, this sterol does not exhibit the absorption maximum at 233 nm reported by Longley et al. (22), and we were therefore unable to identify it. Table 3 also shows that the proportions of the two major sterols differ only slightly in vegetative cells and in cells incubated in sporulation medium for 24 and 120 hr. No significant differences in the proportions of the two major sterols were recorded when the saponification assay method was used instead of extraction with chloroform-methanol. Sterols in the extracts, which include those present in cells in the free and esterified form, were assayed by using the Liebermann-Burchard and Shaw and Jefferies methods (Table 4). The Shaw and Jefferies method assays only ergosterol. The amounts of sterol assayed by this method (Table 4), when expressed as a percentage of the sterol assayed by the Liebermann-Burchard method, are very similar to the percentage of ergosterol assayed by gas-liquid chromatography.

Incorporation of radioactive acetate into lipid fractions. When acetate-*U*-^{14}C was included in the sporulation medium, about half of the label incorporated into cells was recovered in the lipids (Fig. 2). This percentage was considerably greater than that reported by the Espositos and their colleagues (11), who used acetate-2-^{14}C. Table 5 shows that the amount of acetate carbon in each of the major lipid fractions during incubation of cells in sporulation medium containing acetate-*U*-^{14}C. During ascus formation, the amounts of carbon, derived from acetate, incorporated into various lipid fractions, increased. However, in lipids from asci harvested after 120 hr of incubation, the contribution of acetate carbon to each of the fractions varied (compare Tables 1 and 5). Over 90% of the weight of sterol esters can be accounted for by acetate carbon, whereas only

50% of the weight of triacylglycerols and 30% of the total phospholipid are similarly accountable. When labeled acetate was added to sporulating cultures over 6-hr periods (Table 6), there was an initial rapid incorporation of label into all of the fractions over the first 6 hr. This rate of incorporation then progressively decreased up to 24 hr. From 24 to 30 hr, there was a further marked increase in incorporation into each of the fractions, but immediately after this period the rate of incorporation fell rapidly.

TABLE 4. *Total sterol contents of developing asci of Saccharomyces cerevisiae DCL 740*

Period in sporulation medium (hr)	Assay method	Sterol content in extracts obtained by[a]	
		Saponification	Extraction with $CHCl_3$-MeOH
0	Liebermann-Burchard	0.16 ± 0.04	0.10 ± 0.03
	Shaw & Jefferies	0.12 ± 0.05	
24	Liebermann-Burchard	0.79 ± 0.05	0.81 ± 0.19
	Shaw & Jefferies	0.32 ± 0.04	
120	Liebermann-Burchard	1.82 ± 0.76	2.23 ± 0.28
	Shaw & Jefferies	1.17 ± 0.42	

[a] Values quoted are milligrams per 10^9 cells with 95% confidence limits.

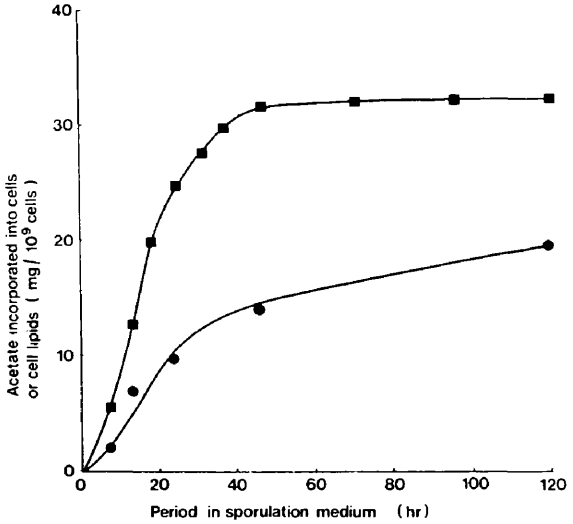


FIG. 2. *Time-course of incorporation of acetate into cells (■) and into cell lipids (●) during incubation of Saccharomyces cerevisiae DCL 740 in sporulation medium containing acetate-U-^{14}C.*

Changes in fine structure during ascus development. Few changes occurred in the fine structure of developing asci during the first 12 hr of incubation, with the exception of an increase in the number of mitochondria, alignment of endoplasmic reticulum around the cell periphery, and fragmentation of the vacuole (Fig. 3, 4, and 5). Incubation for another 6 hr led to more dramatic changes. Fragmentation of the central vacuole gave rise to many dispersed vacuoles, but the most notable features were the presence of numerous electron-transparent vesicles and the formation of extensive endoplasmic reticulum membranes (Fig. 6). The process of ascospore formation was rapid and apparently asynchronous since a variety of developmental stages were found in cells harvested after 24 hr of incubation in sporulation medium (Fig. 7–11). During this phase, the nucleus changed shape extensively, probably reflecting a state of division (compare Fig. 7 and 8). As this process continued, a double, delimiting membrane extended around the extremity of each lobe of the nucleus (Fig. 8). Electron-transparent vesicles became aggregated around these delimiting membranes, especially when adjacent lobes were close together (Fig. 8 and 9). Subsequently, many of the vesicles became enclosed within the delimited ascospores (Fig. 9, 10, and 11), around which walls were developed (Fig. 10 and 11).

DISCUSSION

Although it has been claimed that asci of *S. cerevisiae* are richer in lipids than vegetative cells (17, 18, 23, 32), these claims are based entirely on cytological observations. The present paper reports for the first time the extent

TABLE 5. *Incorporation of acetate carbon into different lipid fractions of Saccharomyces cerevisiae DCL 740 during ascus formation*[a]

Period in sporulation medium (hr)	Phospholipid	Sterol	Sterol ester	Diacylglycerol	Triacylglycerol	Free fatty acid	Total
6	0.36 ± 0.02	0.05 ± 0.00	0.27 ± 0.03	0.04 ± 0.00	0.21 ± 0.01	0.03 ± 0.01	0.98 ± 0.04
12	0.58 ± 0.05	0.08 ± 0.01	0.74 ± 0.16	0.14 ± 0.01	0.81 ± 0.23	0.11 ± 0.01	2.51 ± 0.04
24	0.72 ± 0.06	0.13 ± 0.00	1.21 ± 0.10	0.07 ± 0.02	0.92 ± 0.07	0.13 ± 0.03	3.15 ± 0.12
48	1.17 ± 0.41	0.21 ± 0.04	1.82 ± 0.15	0.22 ± 0.02	1.37 ± 0.14	0.39 ± 0.09	5.19 ± 0.64
120	0.78 ± 0.02	0.43 ± 0.04	2.80 ± 0.07	0.17 ± 0.04	2.58 ± 0.07	0.29 ± 0.03	7.05 ± 0.19

[a] Values quoted are milligrams of carbon incorporated into each lipid fraction from 10^9 cells, with 95% confidence limits, when acetate-U-^{14}C was included in the sporulation medium.

TABLE 6. *Pulse-labeling of lipid fractions of Saccharomyces cerevisiae DCL 740 following addition of acetate-U-^{14}C to sporulation cultures*[a]

Period during which ^{14}C-acetate was in sporulation culture[b]	Phospho-lipid	Sterol	Sterol ester	Diacyl-glycerol	Triacyl-glycerol	Free fatty acid	Total
6–12	0.34 ± 0.03	0.38 ± 0.23	0.36 ± 0.02	0.04 ± 0.01	0.61 ± 0.06	0.05 ± 0.01	1.46 ± 0.04
12–18	0.33 ± 0.02	0.03 ± 0.17	0.30 ± 0.09	0.03 ± 0.04	0.62 ± 0.16	0.09 ± 0.01	1.23 ± 0.20
18–24	0.24 ± 0.02	0.03 ± 0.00	0.30 ± 0.02	0.03 ± 0.00	0.38 ± 0.04	0.04 ± 0.02	1.01 ± 0.07
24–30	0.42 ± 0.03	0.10 ± 0.01	0.89 ± 0.02	0.10 ± 0.01	0.90 ± 0.09	0.16 ± 0.01	2.57 ± 0.10
30–36	0.14 ± 0.02	0.02 ± 0.01	0.19 ± 0.09	0.04 ± 0.01	0.18 ± 0.02	0.01 ± 0.01	0.59 ± 0.06
36–42	0.16 ± 0.01	0.03 ± 0.00	0.23 ± 0.02	0.04 ± 0.01	0.23 ± 0.01	0.04 ± 0.01	0.72 ± 0.08
114–120	0.05 ± 0.01	0.02 ± 0.00	0.10 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.00	0.28 ± 0.01

[a] Values quoted are milligrams of carbon incorporated into each fraction from 10^9 cells with 95% confidence limits.

[b] Labeled acetate was added to the sporulating culture at the time indicated, and cells and asci were harvested 6 hr later.

and nature of the changes in lipid composition that occur when diploid cells of *S. cerevisiae* form asci containing four haploid ascospores.

The main lipids in the plasma membrane of *S. cerevisiae* are phospholipids and sterols (K. Hunter, thesis, University of Bath). Calculations show that the formation of four ascospores in asci of *S. cerevisiae* DCL 740 (based on average dimensions from our electron micrographs) involves the formation of approximately 80 to 100% more membrane as a result of the formation of a plasma membrane and a spore membrane in each ascospore. The increase in the content of free sterol during the first 24 hr of ascus development could therefore be accounted for very approximately by the formation of new membranes. However, over the same period, the increase in phospholipid content of developing asci is greater than that required to make additional plasma membrane and spore membrane. Some of this phospholipid may be incorporated into newly formed mitochondria, as well as into membraneous structures that are retained in the ascan epiplasm. Some may also be present in membranes that enclose the newly synthesized electron-transparent vesicles. The electron micrographs suggest that a membrane of this type may be present.

The really dramatic changes in the lipid composition of developing asci are in the contents of sterol esters and triacylglycerols. Little is known of the cellular location and physiological significance of these lipids in yeast, but it has been shown that sterol esters and triacylglycerols are mainly located in *S. cerevisiae* in low-density structures (G. E. Wheeler, *unpublished observations;* K. Hunter, thesis, University of Bath) similar to those found in liver cells (25). It seemed possible that the increased synthesis of sterol esters and triacylglycerols might be associated with the formation of additional electron-transparent vesicles during ascus development. Our analytical data show that the contents of both classes of lipid increase steadily during ascus development, whereas the electron micrographs reveal that the formation of electron-transparent vesicles is rapid during the first 24 hr incubation, after which it levels off; indeed, it appears that there may be less vesicular material in asci at later stages of development. Clearly, therefore, the appearance of vesicles cannot be closely correlated with the contents of sterol esters and triacylglycerols in asci, and it is presumed that, if the vesicles do contain these classes of lipid, some must also be located in other ascan structures during the later stages of development.

It is possible only to speculate on the physiological role of the increased production of sterol esters and triacylglycerols some of which may be in vesicles. Vesicles of the type shown in the present study to be formed during ascus development have been implicated in secretion of enzymes (3) and with the extrusion of cell wall material (35) in vegetative cells of *S. cerevisiae*, although in neither study were the vesicles isolated or their chemical compositions determined. One possible role for the vesicles in ascus development is in the formation of the spore wall either by supplying previously synthesized wall material or by acting as a repository of wall-synthesizing enzymes. This notion is supported by two findings: firstly, the very rapid incorporation of acetate into sterol esters and triacylglycerols during the period (24–30 hr) during which spore walls are being formed;

FIG. 3. *Vegetative cell of Saccharomyces cerevisiae DCL 740. The nucleus (N) and the tonoplast of the tangentially sectioned vacuole (V) occupy the central region of the cell. A few mitochondria (M) and membrane profiles of endoplasmic reticulum (ER) occur around the periphery of the cell. Vertical bar represents 1 μm.*

FIG. 4. *Developing ascus after 6 hr of incubation in sporulation medium showing few structural changes from the vegetative phase except for an increase in the number of mitochondria (M) and the alignment of endoplasmic reticulum (ER) around the periphery of the cell. Vertical bar represents 1 μm.*

FIG. 5. *Developing ascus after 12 hr of incubation in sporulation medium. The vacuole (V) is highly lobed as seen in tangential section, and a few electron-transparent vesicles (TV) bounded by a dense layer are seen in the cytoplasm. Vertical bar represents 1 μm.*

FIG. 6. *An ascus after 18 hr of incubation in sporulation medium showing dramatic changes in cellular fine structure. Numerous electron-transparent vesicles (TV) can be seen throughout the cell, an extensive membrane system is present (ER), and the vacuole has now fragmented, resulting in several scattered vacuoles (V) some of which contain small electron-transparent vesicles. Vertical bar represents 1 μm.*

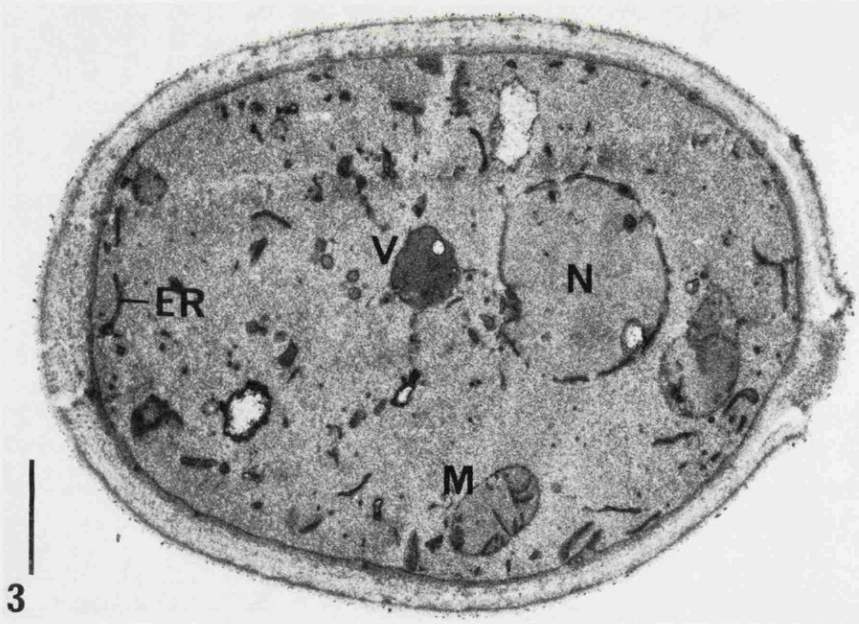
FIG. 7. *An ascus after 24 hr of incubation in sporulation medium. Numerous electron-transparent vesicles (TV), vacuoles (V), and endoplasmic reticulum profiles (ER) are present throughout the cell. Verticle bar represents 1 μm.*

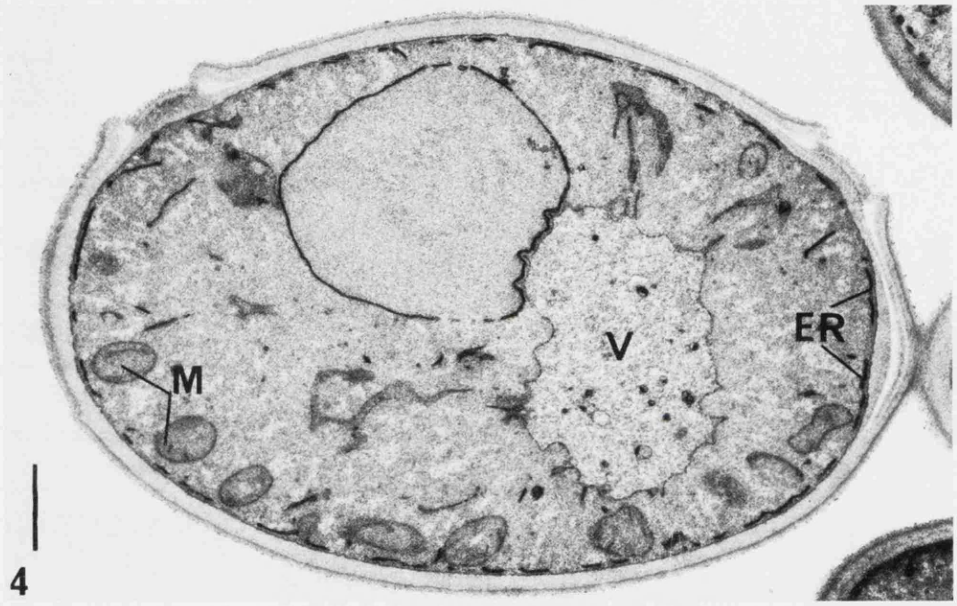
FIG. 8. *An ascus after 24 hr of incubation in sporulation medium. The highly lobed nucleus (N) is apparently undergoing division, and the spore-delimiting membranes (SDM) can be seen enveloping the lobes of the nucleus in what are assumed to be the polar regions of the intranuclear spindle. The spindle microtubules are not preserved by potassium permanganate fixation. Electron-transparent vesicles (TV) are aligned around and between the lobes of the nucleus. Vertical bar represents 1 μm.*

FIG. 9. *Another ascus after 24 hr of incubation in sporulation medium. Ascospore delimitation is nearing completion. Electron-transparent vesicles (TV) are enclosed within the spore-delimiting membranes (SDM). Smaller vesicles with electron-transparent centers and thick, dense bounding layers are closely aligned between the delimiting spores. Vertical bar represents 1 μm.*

FIG. 10. *Another ascus after 24 hr of incubation in sporulation medium. Delimitation of ascospores (AS) has just reached completion. Electron-transparent vesicles (TV) are present both within the spores and in the ascus cytoplasm. Vertical bar represents 1 μm.*

FIG. 11. *Another ascus after 24 hr of incubation in sporulation medium. Four ascospores (AS) are visible, and around each an ascospore wall (ASW) has developed. Vertical bar represents 1 μm.*





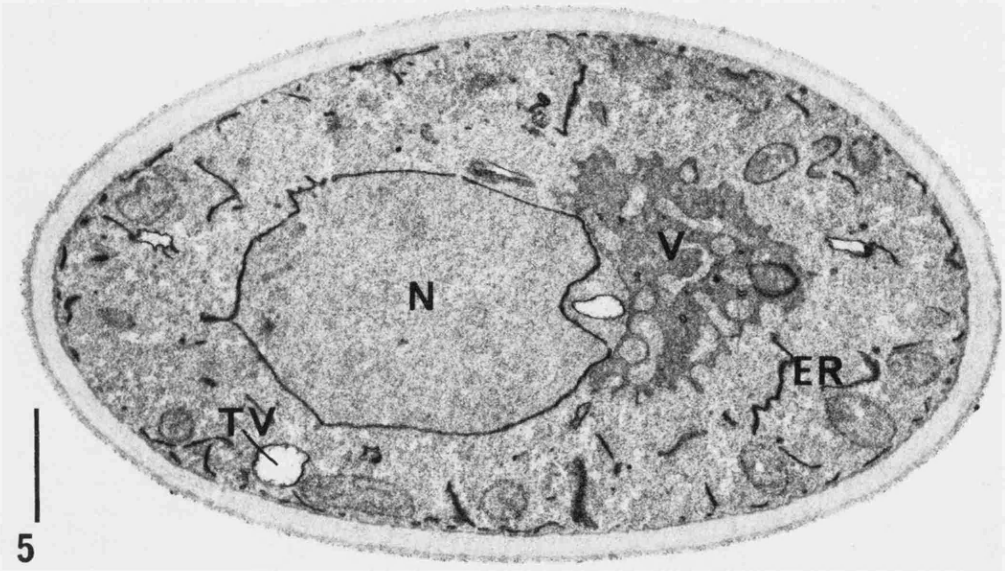
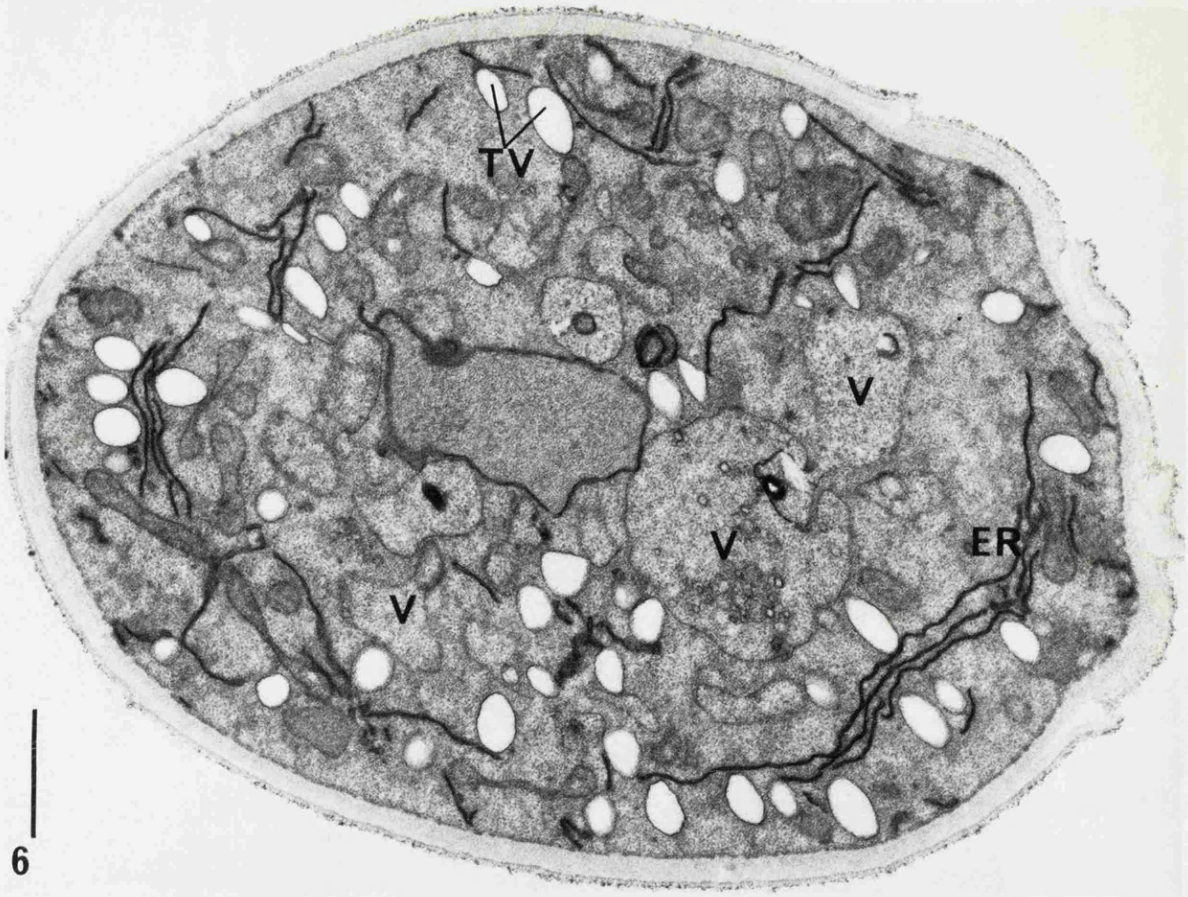


FIG. 3–5



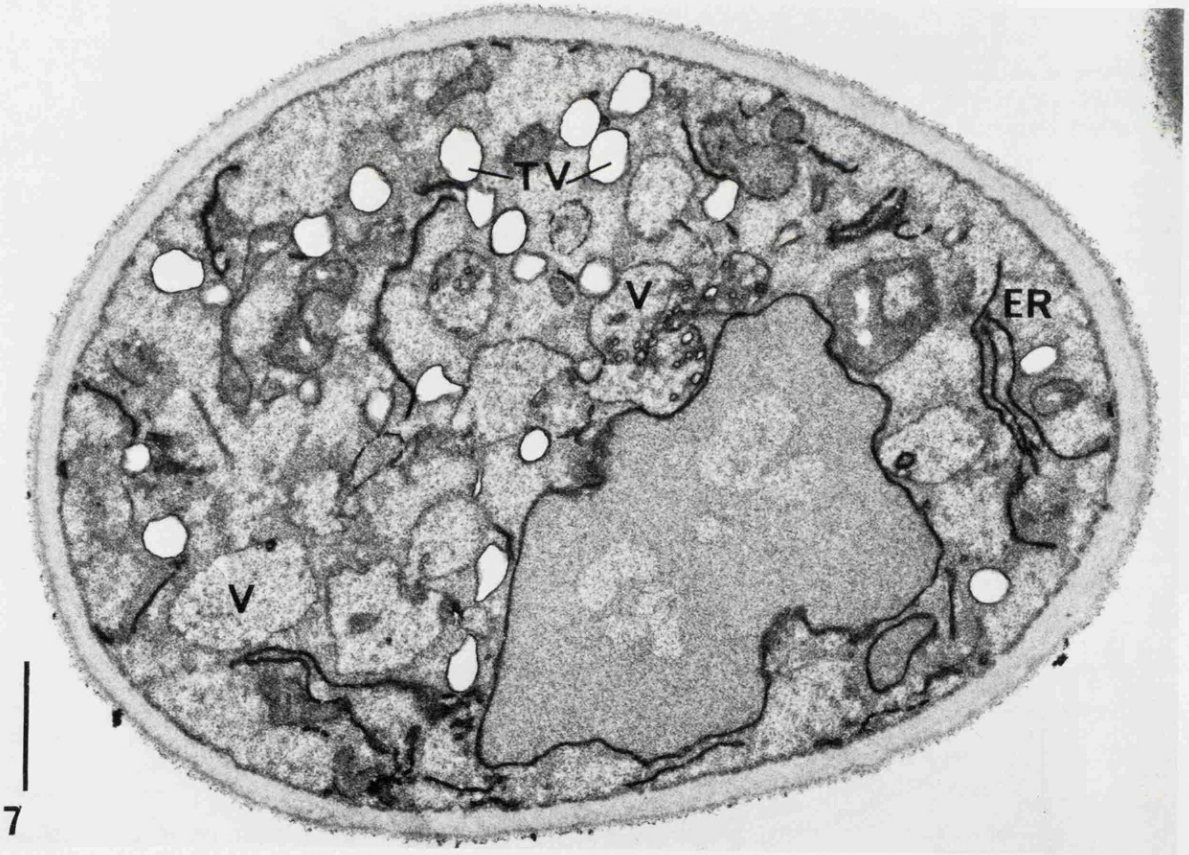
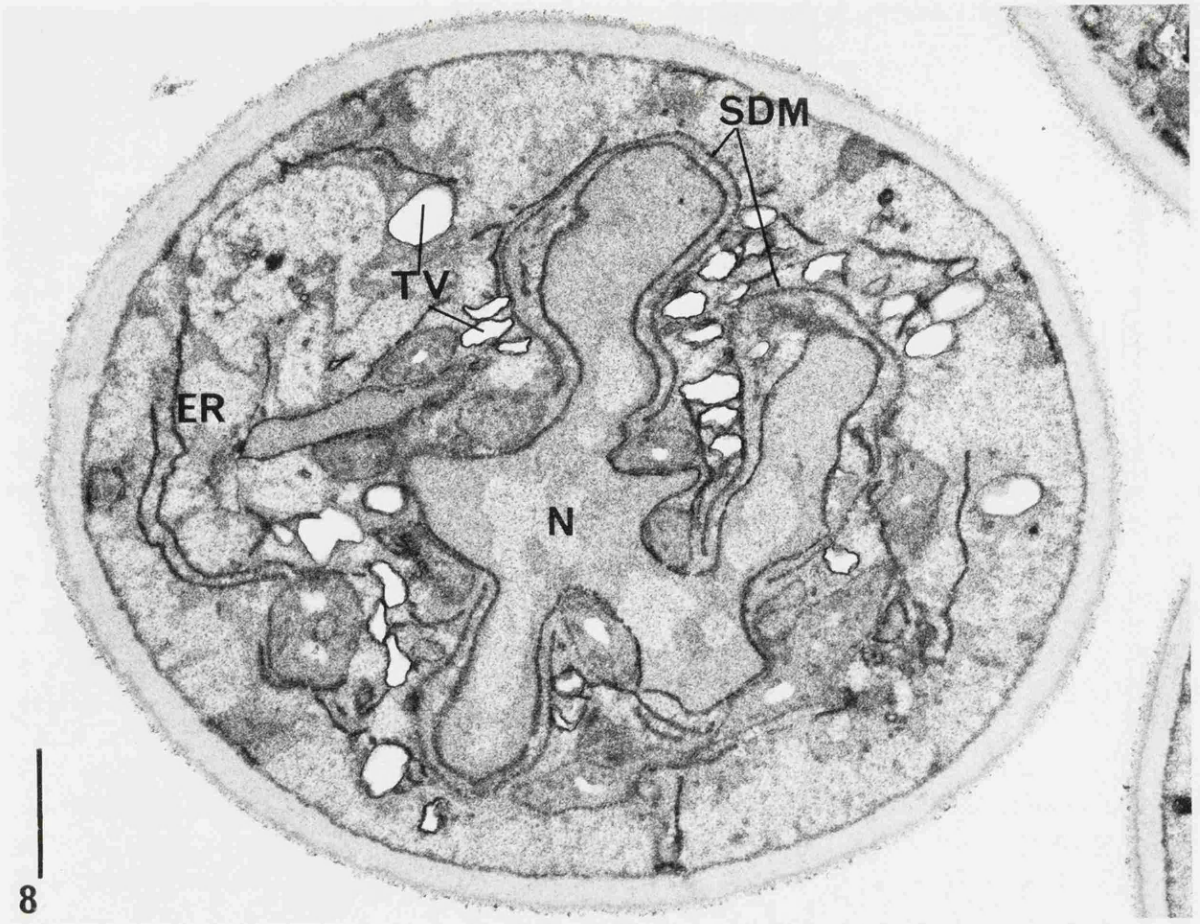


FIG. 6 AND 7



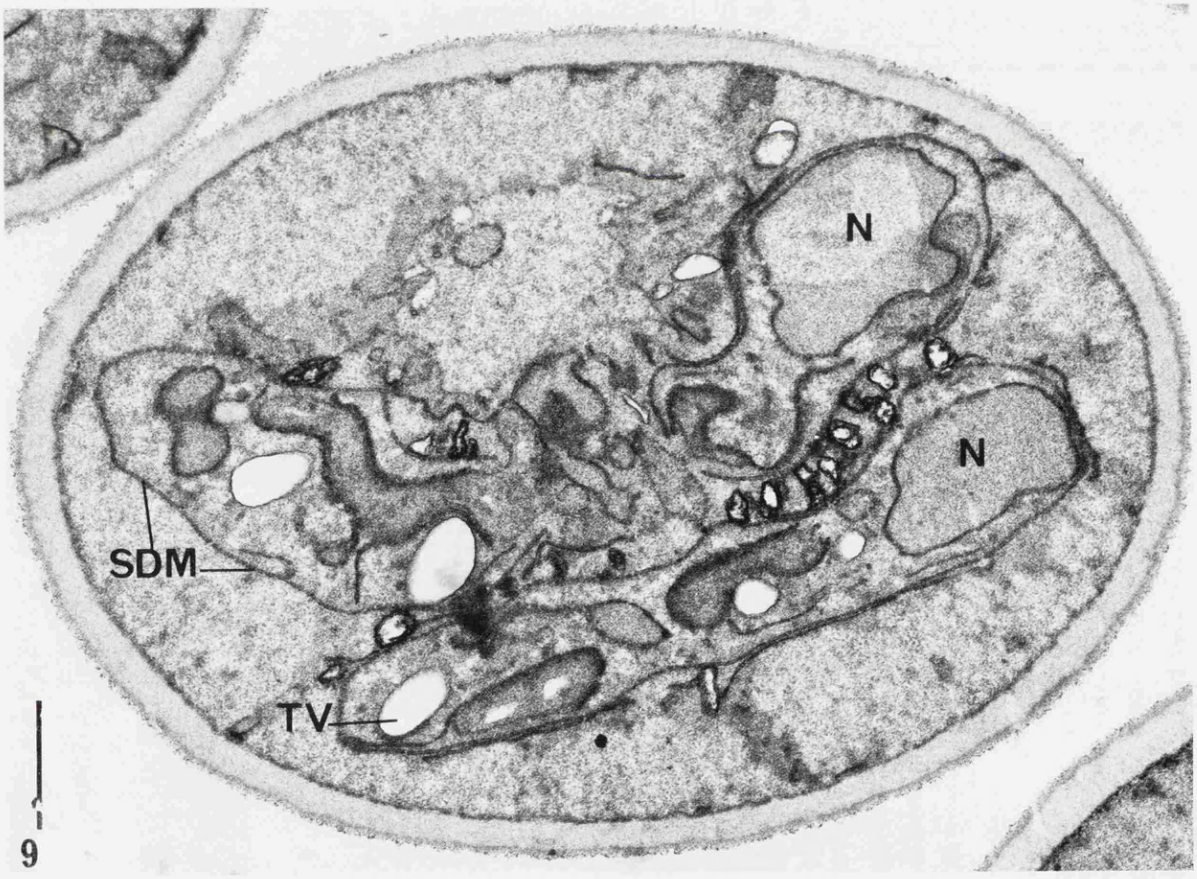
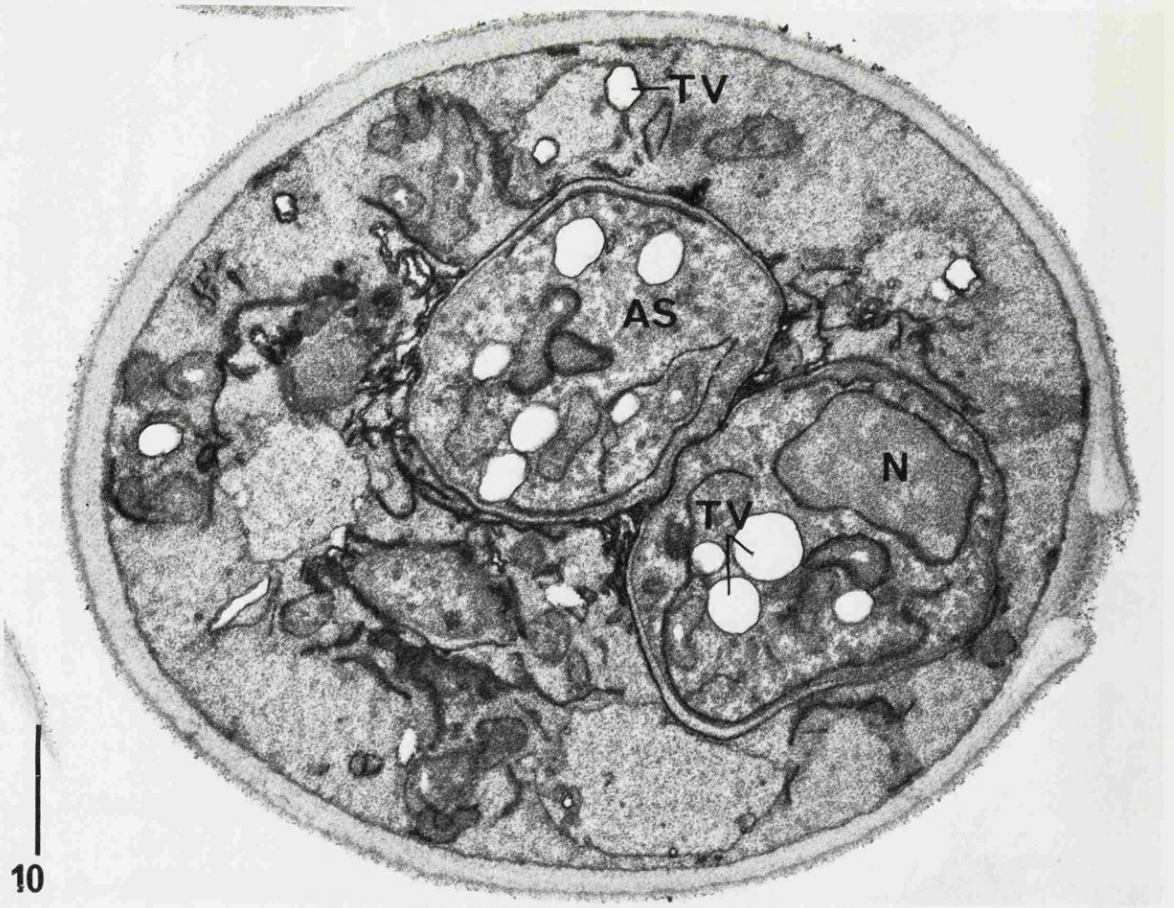


FIG. 8 AND 9



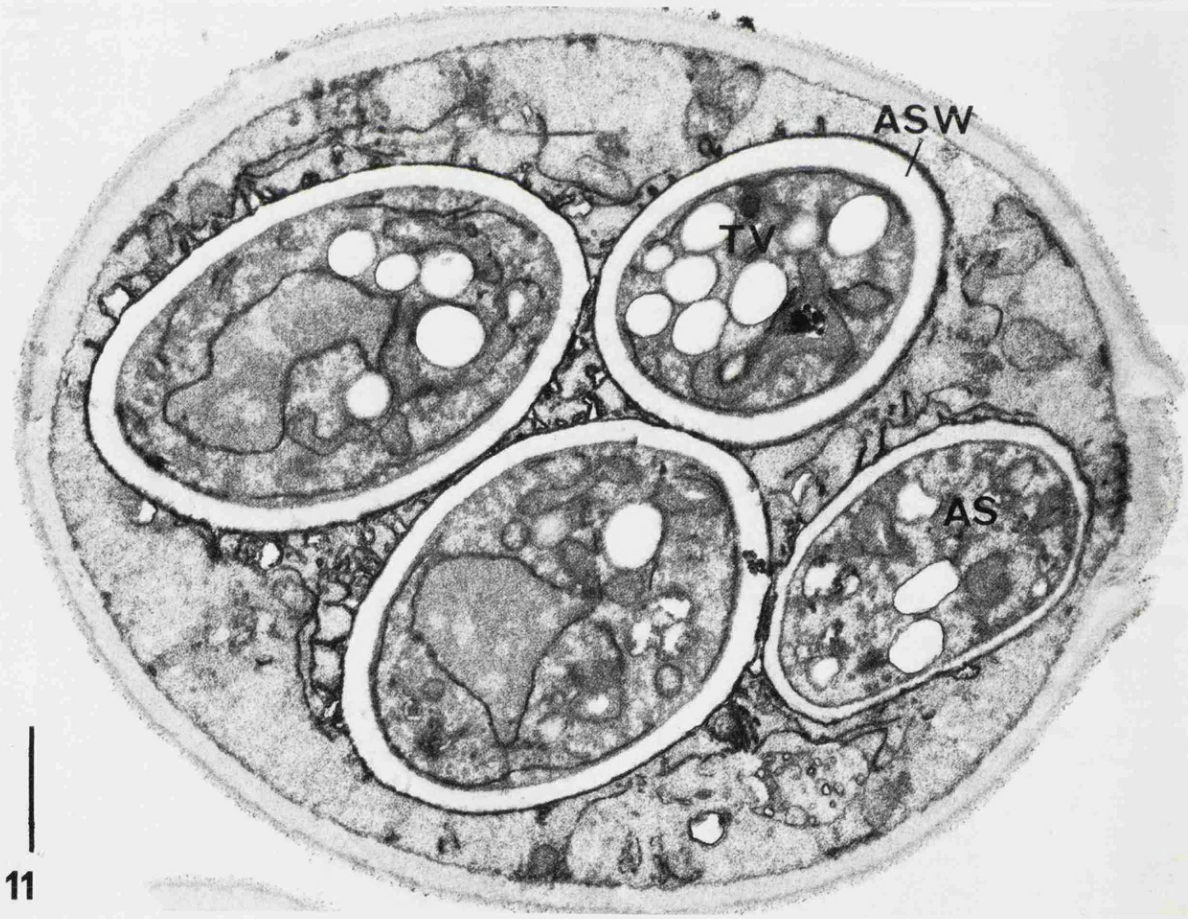


FIG. 10 AND 11

secondly, the location of the electron-transparent vesicles in asci at the time of spore delimitation. A similar association between osmiophilic (lipid) vesicles and "prospore wall" was demonstrated by Moens (26) in glutaraldehyde-fixed cells of *S. cerevisiae* although Moens does not discuss the possibility of a role for these vesicles in wall synthesis. Although our data do not indicate the preferential location, if any, of unsaturated fatty acids in ascan lipids, it is conceivable that these acids are concentrated in sterol esters and triacylglycerols where the extra mobility which unsaturated fatty acids confer on lipids (12) might be important in vesicles which transport wall material or enzymes. However, the increased synthesis of sterol esters and triacylglycerols, both of which are hydrophobic in nature, might be expected to lower the water-retaining capacity of asci, a suggestion which makes it difficult to explain the observed increase in water-retaining capacity of asci as development proceeds.

Electron micrographs obtained in the present study also show that the nucleus becomes highly lobed during meiosis. This finding is in agreement with the observations of Moens and Rapport (27) on "uninuclear meiosis," in which meiosis I and meiosis II are thought to take place without rupture of the nuclear membrane. These observations have recently been confirmed by the elegant freeze-etched micrographs published by Conti and his colleagues (16). Also worthy of mention is the presence of double, delimiting membranes, and their alignment around the meiotic nucleus. The inner of these membranes would appear to form the ascospore plasma membrane. By analogy with other ascomycetes (2, 6, 15, 24, 31), it is presumed that the spore wall is formed by insertion of material between these membranes. This essentially supports the findings of Lynn and Magee (23) with *S. cerevisiae* and Black and Gorman with *Hansenula wingei* (4). The outer membrane presumably gives rise to the proteinaceous spore membrane (5).

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Ascospore Wall Development in *Saccharomyces cerevisiae*

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Ascospore delimitation in *Saccharomyces cerevisiae* is initiated by a pair of unit membranes between which the spore wall is subsequently laid down.

It has been claimed that during sporulation in *Saccharomyces cerevisiae* a single membrane, which eventually becomes the spore plasma membrane, delimits individual ascospores, and that spore wall material is deposited around this membrane (6). Other reports, based on examination of sectioned material fixed with glutaraldehyde (10) or of replicas of freeze-etched material (5), claim that individual ascospores are delimited by a bilaminar structure, variously referred to as the "prospore wall" (10, 11) or the "forespore membranes" (5), which gives rise to the spore wall in the mature ascospore. In this context Lynn and Magee (8) and Marquardt (10) referred to a spore envelope composed of two unit membranes between which the spore wall material is formed.

We have presented evidence (7) supporting the contention made by Lynn and Magee, and by Marquardt, namely that a double-unit membrane rather than a single membrane initiates delimitation of the ascospores, and we further suggested that the spore wall develops by insertion of material between these two membranes. This note presents further evidence to support the involvement of a double membrane during ascosporogenesis, and demonstrates participation of lipid vesicles in spore wall formation.

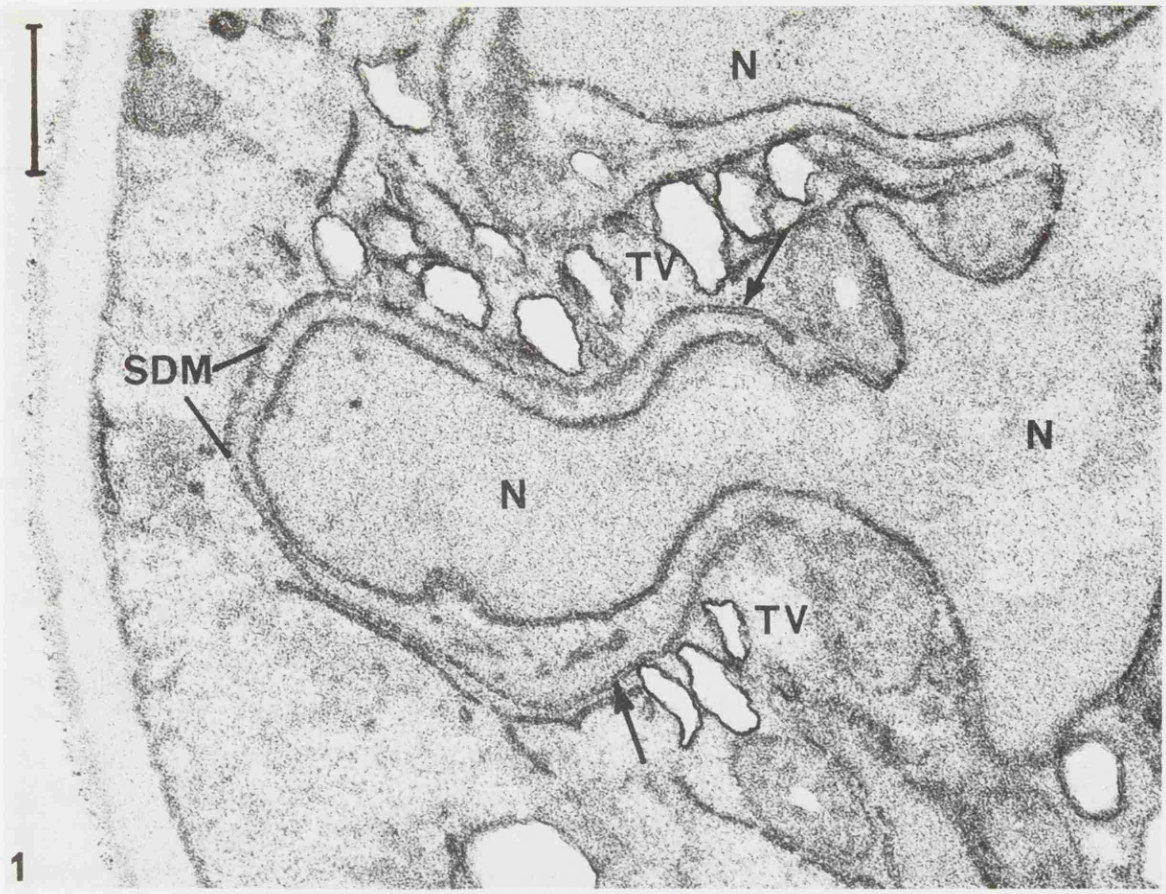
A strain of baker's yeast, *S. cerevisiae* DCL 740, was used both in this and previous studies (3, 7). Methods for preparing material for electron microscopy were as reported elsewhere (7).

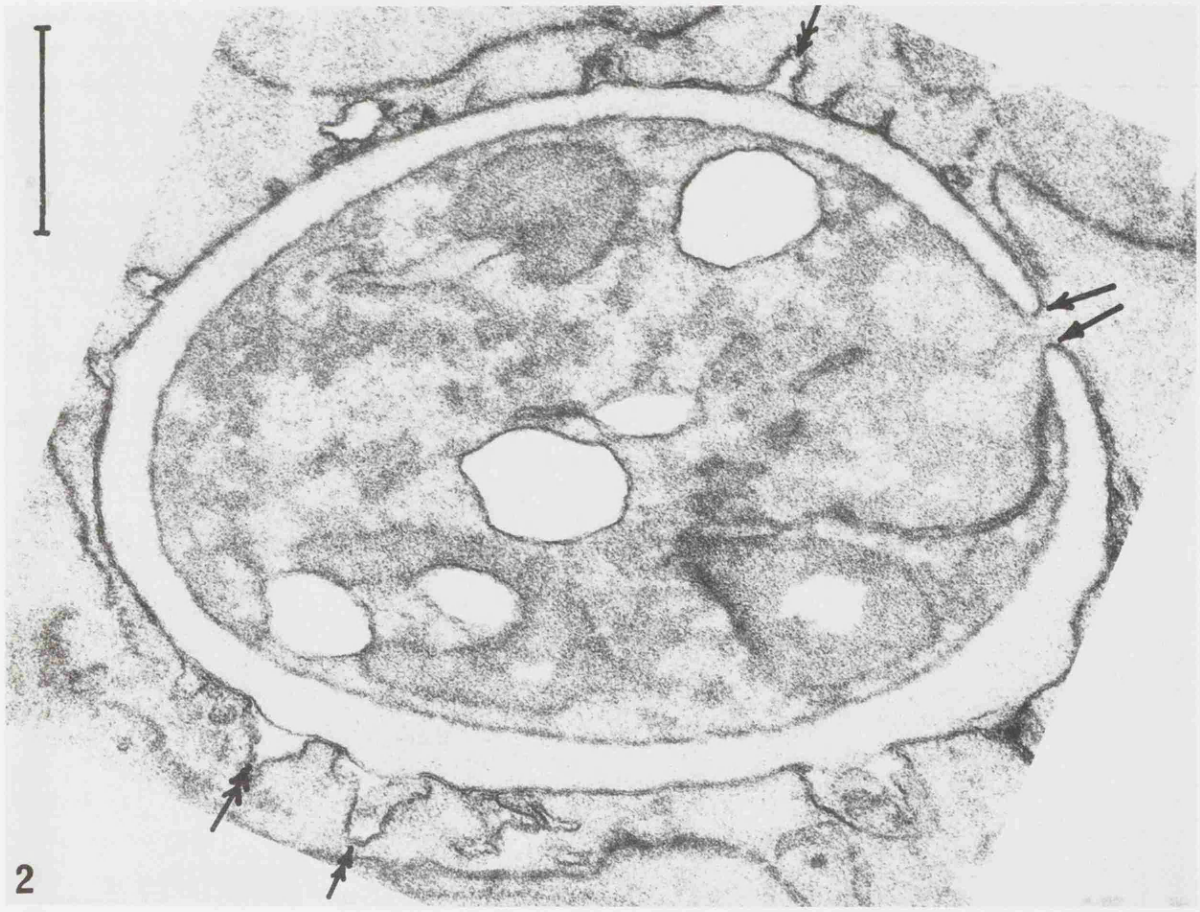
Figure 1 shows lobes of the meiotic nucleus after 24 hr of incubation in sporulation medium. Both lobes are partially enveloped by the spore-delimiting membranes, which, like the nuclear membrane, are comprised of two parallel membranes. The association between the spore-delimiting membranes and the lipid vesicles, discussed previously (7) and noted by Lynn and Magee (8), is evident. A section through an immature spore is shown in Fig. 2. This micrograph reveals that the spore-delimiting membranes have failed to envelop the spore nucleus completely, leaving a small pore, at which point the two unit membranes are seen to be continuous. A certain amount of spore wall material has already been deposited in the space between these two membranes, a process which continues as the spore wall matures. Before the spore wall develops, the two membranes appear to be equally dense, but, as the spore matures, the outer spore-investing membrane becomes more heavily stained, whereas the inner membrane, now the spore plasma membrane, is less stained (cf Fig. 1, 2, and 3).

Several electron-transparent blebs are visible on the outer spore-investing membrane (Fig. 2). Figure 3 shows a similar bleb in detail. These blebs appear to be situated in the electron-transparent middle layer of the unit membrane which swells to many times its

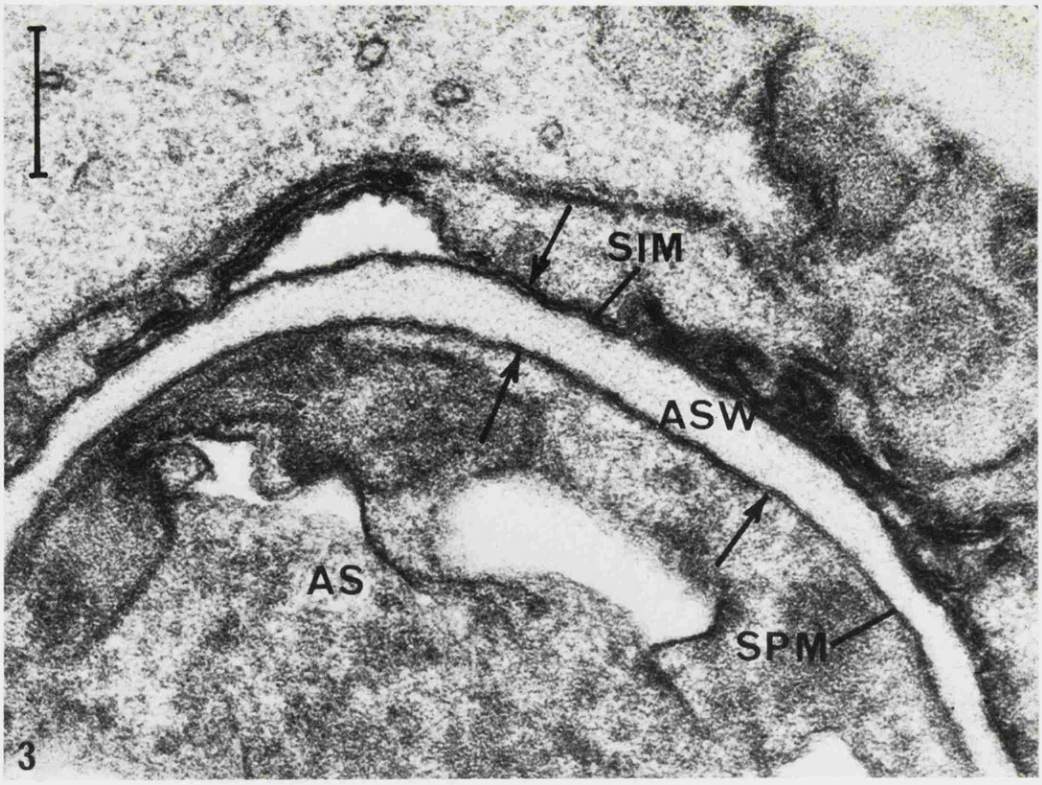
FIG. 1. *Part of a transversely sectioned ascus in which spore-delimiting membranes (SDM) have begun to envelop the lobe of a multilobed, dividing nucleus (N). Transparent vesicles (TV), interpreted as being lipid, are associated with the spore-delimiting membranes which can be seen to be double (arrows). Bar = 0.5 μm.*

FIG. 2. *Transverse section of an incompletely delimited ascospore in which deposition of spore wall material has occurred between the two delimiting membranes which are seen to be continuous (arrows). The close association of transparent (lipid) vesicles with the outer of the two delimiting membranes can be seen (double arrows). Bar = 0.5 μm.*





FIGS. 1 and 2.



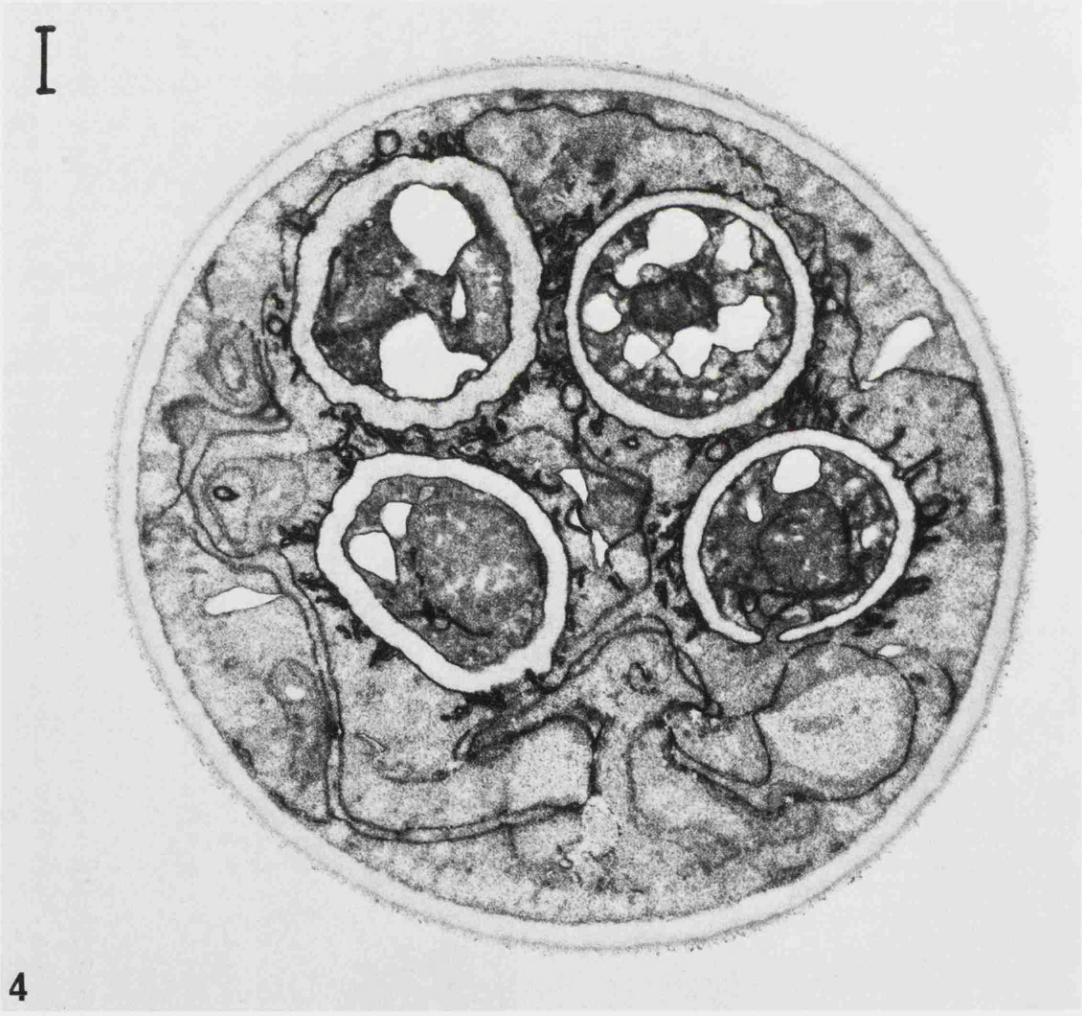


FIG. 3. *Part of a maturing ascospore (AS) showing the unit membrane structure of both the inner, spore plasma membrane (SPM) and the outer, spore-investing membrane (SIM) (arrows). Material of*

original thickness. This middle layer is recognized as being the location of the fatty-acyl residues of the membrane phospholipids and it would therefore seem likely that these blebs are composed mainly of lipid. Possibly the blebs are formed from the lipid vesicles seen in Fig. 1. Their association with the outer membrane supports our hypothesis that the lipid vesicles are involved in spore wall synthesis (7). Figure 4 shows an immature ascus (after 24 hr of incubation in sporulation medium). The surfaces of the ascospores are covered with membraneous debris, possibly the remains of blebs which have emptied and collapsed. The presence of a unit membrane on the surface of developing ascospores (Fig. 2, 3) corroborates our earlier finding that mature ascospores of *S. cerevisiae* possess a surface protein layer (3).

The spore-delimiting membranes described in this paper are equivalent to both the prospore wall described by Moens (11, 12) and the forespore membranes described by Guth et al. (5). Each of these structures is bilaminar and demarcates a spore by progressive extension around a lobe of the post-meiotic nucleus. Delimitation of ascospores and spore wall development in species of higher ascomycetes are chronicled elsewhere (1, 2, 4, 9, 13). Guth et al. (5) point out that the origin of the spore-delimiting membranes is obscure in *S. cerevisiae*, whereas, in certain higher ascomycetes, spore-delimiting membranes [which in *Saccobolus* and *Ascodesmis* fuse to form an "ascus vesicle" (4)], arise from membrane-bound vesicles which originate from the nuclear envelope and endoplasmic reticulum (2, 4). This observation led Guth et al. (5) to state: "the process of formation of ascospore wall (in *S. cerevisiae*) is unlike that reported for other ascomycetes. . . ." We believe this conclusion to be mistaken and we assert that, firstly, the spore-delimiting membranes in *S. cerevisiae* and the ascus vesicle in higher ascomycetes are essentially similar both in structure and function; secondly, the process of spore wall development between two opposing membranes is very similar in both instances.

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the ascospore wall (ASW) has been deposited between these two membranes, and a lipid vesicle can be seen closely associated with the spore-investing membrane. Bar = 0.25 μm.

FIG. 4. *Transverse section of an ascus containing four maturing ascospores. Around the outside of each ascospore is a mass of membraneous debris probably derived from collapsed lipid vesicles which have been incorporated in the developing spore wall. Bar = 0.5 μm.*